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Escherichia coli adenlyosuccinate synthetase: studies on substrate specificity

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A dissertation submitted to the graduate faculty
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has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

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For the Major Program

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NOMENCLATURE

AMPSase	- adenylosuccinate synthetase, *Escherichia coli*
AdSS1	- adenylosuccinate synthetase, mouse muscle, basic
AdSS2	- adenylosuccinate synthetase, mouse non-muscle, acidic
AMP	- adenine monophosphate
AMPS Lyase	- adenylosuccinate lyase
ASP	- L-aspartate
ATP	- adenine triphosphate
CD	- circular dichroism
E. coli	- *Escherichia coli*
HPLC	- high performance liquid chromatography
IMP	- inosine monophosphate
k_{cat}	- catalytic constant
K_m	- Michaelis constant
K_d	- dissociation constant
KP_i	- potassium phosphate
MALDI	- matrix-assisted laser desorption ionization
PCR	- polymerase chain reaction
P_i	- orthophosphate
SDS-PAGE	- sodium dodecyl sulfate polyacrylamide gel electophoresis

CHAPTER I. GENERAL INTRODUCTION

Enzymes are catalysts of biological reactions, and are characterized by their substrate specificity, specificity of reaction and ability to be regulated. The purpose behind enzymatic studies is to determine what chemical reaction is catalyzed, and how the structural features of an enzyme are responsible for a particular function. The aspects of enzymes studied are the catalytic mechanism of the enzyme, the association states of the enzyme, the constituents of substrate specificity and the regulation of the enzyme. Studies such as these are called structure-function studies.

By changing specific interactions and determining how this changes the action of the enzyme, we can determine what specific interactions are responsible for specific functions. This can yield the mechanisms for inactivation of the enzyme, as well as the design of more potent agonists, antagonists and inhibitors. This knowledge can be extrapolated to define actions of enzymes whose function may be known but have not been completely characterized or crystallized. This information could also be used to help predict the action of proteins which are postulated from genes in the various gene databases resulting from the genome sequencing projects, for example the *Saccharomyces cervesiae*, *Drosophia melanogaster* and *Helicobacter pylori* genomes.

One enzyme studied as a model system in our laboratory is adenylosuccinate synthetase (E.C. 6.3.4.4, L-aspartate:IMP ligase, GDP forming; AMPSase). This enzyme is the first committed step in the formation of adenine monophosphate (AMP) from the branch point of inosine monophosphate (IMP) in *de novo* purine nucleotide biosynthesis. The pool of adenine nucleotides is of vital importance for both cellular replication and cellular energy

stores. Due to this importance, AMPSase is essential for cellular growth. Indeed, there has been no organism yet found that does not have an isozyme of AMPSase. It has also been found to have changes in disease states, such as acute lymphomatic leukemia (1), to be a target of natural antibiotics (2) and a proherbicide (3).

The role of specific interactions at the interface of the enzyme dimer, and the roles of specific interactions between the enzyme and substrates in the active site of the enzyme are the focus of this dissertation. Interactions were studied by producing site-directed mutants of AMPSase as guided by comparison of the unligated and ligated crystal structures. The wild-type and mutant AMPSases were then characterized by biochemical and biophysical approaches. These included determination of their kinetic parameters utilizing spectrophotometric assays, and study of their global conformations by circular dichroism (CD), analytical ultracentrifugation, and matrix-assisted laser desorption ionization (MALDI) studies.

Thesis Organization

This thesis is a general introduction and review of the relevant literature, followed by a series of manuscripts, and a general conclusion. Studies on the interactions of specific residues at the interface of the AMPSase dimer, and the implications the dimeric form of the enzyme has on the action and regulation of the enzyme are the focus of Chapter II. Chapter III examines the interactions between a group of side-chain residues and the 5'-phosphoryl group of IMP in the active site near one dynamic loop of the enzyme. The expected and unexpected results to binding of IMP and catalytic activity of the enzyme are discussed. A group of β-branched side-chains is focus of Chapter IV, which discusses their role in the

determination of substrate binding of IMP and L-aspartate. The role of Val^{273} in determining the specificity of the L-aspartate binding site, though there are no direct interactions between it and L-aspartate found in the crystal structure of the enzyme, is examined.

Literature Review

In 1955, inosine monophosphate (IMP) was first recognized as a precursor of adenine monophosphate (AMP) in rabbit bone marrow extracts by Abrahams and Bently (4) and also by Carter and Cohen from yeast extracts (5). Carter and Cohen also recognized an intermediate product is involved in this pathway, and were able to purify and identify this intermediate as adenylosuccinate (adenylosuccinic acid, AMPS) (6). Soon afterwards, Lieberman showed that adenylosuccinate could be formed from IMP in *Escherichia coli* (*E. coli*) extracts. Additionally, he determined the nitrogen source of the reaction was L-aspartate (ASP), not ammonia as found in the *de novo* biosynthetic pathway for pyrimidines, and the energy source of the reaction was guanine triphosphate (GTP) (7). The overall reaction described is:

$$\text{L-aspartate} + \text{IMP} + \text{GTP} \xleftrightarrow{Mg^{2+}} \text{adenylosuccinate} + \text{GDP} + \text{Pi} \qquad \text{(Reaction 1)}$$

The enzyme which catalyzes this reaction is adenylosuccinate synthetase [E.C. 6.3.4.4, IMP:L-aspartate ligase, GDP forming, AMPSase]. Adenylosuccinate is formed from the addition of aspartate to the C6 position on the purine ring of IMP, with the hydrolysis of the γ-phosphate of GTP. In the final step of *de novo* pathway of AMP synthesis from IMP, adenylosuccinate is cleaved by the enzyme adenylosuccinate lyase, forming AMP and fumerate. Adenylosuccinate synthetase is an essential enzyme when no extraneous source of

adenine is present; i.e. when AMP cannot be formed through salvage pathways. With the exception of human erythrocytes, it has been found in all organisms and tissues studied (8). Numerous studies have been undertaken to understand the kinetic and catalytic mechanisms of the reaction, structure of the enzyme and roles specific residues play in substrate binding and catalysis.

Kinetically, the reaction mechanism is sequential rapid-equilibrium random ter-ter (9), though one isotope exchange study has suggested nucleotide binding before L-aspartate binds the enzyme is slightly preferred (10). From 1956 to 1977, three different mechanisms were proposed for the catalysis of Reaction 1 by AMPSase, all of which are consistent with the random ter-ter model. Of these, the most widely accepted mechanism involves the attack of the 6-oxo position of IMP by the γ-phosphoryl group of GTP, forming the intermediate 6-phosphoryl IMP. This is followed by the nucleophillic attack of the 6-phosphoryl IMP by the amino nitrogen of aspartate, resulting in adenylosuccinate, GDP and P_i, as was proposed separately by Lieberman and Fromm (7,11).

The two other mechanisms are a concerted mechanism proposed by Miller and Buchanan, where all three substrates interact simultaneously (12), or IMP-aspartate reaction followed by GTP hydrolysis as proposed by Markham and Reed (13). Support for the exchange of the γ-phosphoryl of GTP to IMP before reaction with aspartate comes from positional isotope exchange studies utilizing [γ-^{18}O] GTP. Isotope exchange occurred only when IMP is present (10). Crystal structures with the presence of 6-thiophosphoryl IMP (14) and 6-phosphoryl-IMP (15) in the active site are additional support. However, no studies performed to date can definitively exclude the remaining two mechanisms.

Early studies used muscle adenylosuccinate synthetase purified mainly from skeletal muscle tissue, as muscle contains a relatively large quantity of the enzyme. In 1977, Matsuda identified a second isozyme of adenylosuccinate synthetase from rat liver tissue (16). The two isozymes have been termed the muscle (M), or basic, isozyme with a pI of 8.9, and non-muscle (L), or acidic, isozyme with a pI of 5.9. The non-muscle isozyme was first identified from liver, thus the L designation (16). The two isozymes have been distinguished upon the basis of their tissue specificity, muscle tissue expressing the basic (M) enzyme exclusively, while other tissues have varying ratios of both isozymes. Besides their tissue localization, the isozymes also differ in their physical parameters, namely their isoelectric points, kinetic parameters, purification and intracellular interactions.

The muscle and non-muscle adenylosuccinate synthetase genes were cloned in 1991 and 1994, and are respectively named AdSS1 and AdSS2 (17,18). Comparison of the deduced amino acid sequences shows 89% similarity and 78% identity between the muscle and non-muscle sequences, performed by BESTFIT (GCG analysis program) (17). Between either AdSS1 or AdSS2 and AMPSase, there is 54% similarity and 43% identity. The non-muscle isozyme, AdSS2, and the *E. coli* enzyme are similar in their pI, purification, and kinetic parameters of the enzyme, so AdSS2 is thought to be biosynthetic in nature, producing the adenine nucleotides necessary for cellular function. The results of a regeneration study support this biosynthetic role for AdSS2. Ikegami and Matsuda reported that after injury to liver tissue, which contains both AdSS1 and AdSS2, the non-muscle to muscle isozyme ratio changes from 60:40 before injury to 80:20 during regeneration (19,20).

A second role exists for adenylosuccinate synthetase in muscle cells, as part of the purine nucleotide cycle (PNC). The cycle is composed of the enzymes adenylosuccinate

synthetase, adenylosuccinate lyase and AMP deaminase as first proposed by Loweinstein in 1972 (21). The cycle involves the reaction of IMP to AMP, through the enzymes AMPSase and adenylosuccinate lyase, then recycling the AMP back to IMP by the enzyme AMP deaminase (Figure 1). The net result of this cycle is the production of fumarate (from the adenylosuccinate lyase reaction) and ammonia (from the AMP deaminase reaction), at the expense of one GTP. The overall net reaction being:

$$\text{L-aspartate} + \text{GTP} + H_2O \longrightarrow \text{fumarate} + \text{GDP} + P_i + NH_3 \qquad \text{(Reaction 2)}$$

The purpose of the cycle has been proposed to be a means of keeping a high ATP/AMP ratio in the cell during periods of exercise, a method of removing ammonia from the intracellular amino acids via aspartate, as well as the production of citric acid cycle intermediates (21).

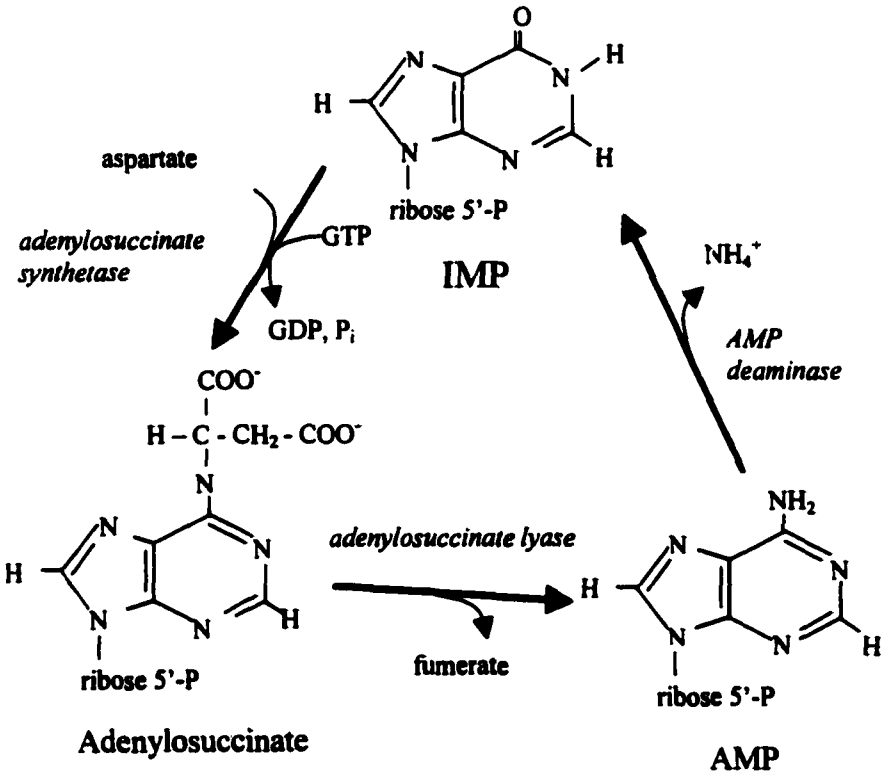

Figure 1. The Purine Nucleotide Cycle, enzymes shown in italics. (after (21)).

The purine nucleotide cycle has been extensively reviewed, and further detail on the cycle can be found elsewhere (21,22). The importance of the PNC in active muscle tissue has been shown through several studies of exercise intolerant patients (patients who experience pronounced fatigue after short periods of energetic activity). In these individuals, AMP deaminase is deficient, and there is a buildup of intracellular concentrations of AMP (23).

As the PNC has been proposed to play an active role in muscle cell metabolism, the muscle isozyme has been associated with the PNC and the non-muscle isozyme has not. While the non-muscle isozyme produces the adenine nucleotides necessary for cellular function, it is thought that the muscle cell produces sufficient AMP for cellular function as the side product of the PNC. These distinctions are due to both the localization and kinetic parameters of the isozymes. Consistent with this hypothesis, the muscle isozyme has been found to have an elevated K_m^{IMP} and slightly decreased K_m^{ASP} and K_m^{GTP} as compared to the acidic isozyme or AMPSase (8). Additionally, high concentrations of IMP have been reported to inhibit the muscle isozyme (8).

As well as kinetic differences between the muscle and non-muscle isozymes, evidence exists for other intracellular differences. The muscle isozyme specifically associates with F-actin, and other actin complexes, while no association is found for the non-muscle isozyme (24-26). The stoichometric ratio of muscle isozyme to F-actin binding is 1 mol of the muscle isozyme to 4 mol of F-actin, with a dissociation constant (K_D) for binding of 0.72 μM. The interaction with F-actin is unique to the basic isozyme, as rat acidic isozyme was not found to associate with F-actin (25). The specificity and level of association between the basic isozyme and actin implies that there is some physiological significance to this interaction, however this is yet to be determined.

The fact that there is an interaction of adenylosuccinate synthetase with other intracellular molecules, such as F-actin, is not unique to the muscle isozyme. *Sacchromyces cervesiae* (*S. cervesiae*) adenylosuccinate synthetase has been found to interact specifically with the T-rich autonomously replicating sequence in the yeast genome (26). A modeled structure for the *S. cervesiae* enzyme suggests the interaction is through a cluster of basic residues on the surface that are not found in other adenylosuccinate synthetases (27). In a recent study, it was suggested that the *S. cervesiae* AMPSase interaction with the ARS sequence is regulated by its state of phosphorylation. While the native purified *S. cervesiae* AMPSase has DNA binding capabilities, a homologously overexpressed and purified *S. cervesiae* AMPSase does not. Additionally, when the native *S. cervesiae* enzyme is dephosphorylated its DNA binding capabilities are lost, while retaining full enzymatic activity (28).

Overexpression of *E. coli* adenylosuccinate synthetase was first achieved in 1987, by Bass *et. al.* (29), and the entire sequence for the *E. coli* adenylosuccinate synthetase gene, *purA*, was first published in 1988, by Wolfe and Smith (30). Since then, sequences for 35 AMPSase genes from unique sources have been described, with identities ranging from 84% to 29% identical to the *E. coli* protein sequence by BLASTP search of the non-redundant Swissprot sequences (31). The first crystallization of any form of adenylosuccinate synthetase was reported in 1977 by Ogawa of the rabbit muscle enzyme (32), however the crystal structure of the enzyme was not solved until 1993 by Poland *et. al.* (33), of the *E. coli* enzyme, with a refined structure of the unligated *E. coli* synthetase reported in 1995 (34). Since then, the structure of the enzyme from *E. coli* complexed with varying ligands and inhibitors has been solved (14,15,35-38). Additionally, the structure has been solved for the

synthetase from three other sources – *Pyrococcus* species (39), *Arabidopsis thaliana* and *Triticum aestivum* (40). The overall three-dimensional structure remains very consistent from all sources, though the *Pyrococcus* species shows some variation, due to its thermophillic source.

The crystallized enzyme is a homodimer with head-to-tail symmetry from all reported sources (35,39,40). Between the unligated and ligated crystal structures there are no global changes, however there are three disordered loops which become ordered upon ligand binding. These loops are known as the 40's loop, the 120's loop and the 300's loop (35,37). Other minor conformational changes occur in the ligated structures, all are minor movements which collapse the structure inwards towards the enzyme active site (35).

Examination of the crystal structures ligated with various ligands can indicate which resides which are potentially important in interactions between the substrates and the enzyme. Resides which are important or essential for catalysis can also be suggested. Using the information gained from the crystal structures, a number of site-directed mutants have been made and characterized. These mutants have been utilized to investigate the substrate-enzyme interactions, mechanistic implications, enzyme-enzyme interface interactions and conformational changes occurring upon substrate binding.

While IMP, GTP and their inhibitors have been crystallized in the active site of the enzyme, only inhibitors of L-aspartate have been successfully crystallized. Thus, good indications of major interactions important for IMP and GTP recognition by the enzyme exist. For example, the guanine binding site was determined to consist of conserved guanine binding residues (N/T/Q)KXD (residues 330-333), and the phosphate binding loop ('P-loop') of GXXXXGK (residues 12-19)(41). Asp^{333} was determined to be an ultimate determinate

for recognition of the guanine base of GTP by the enzyme, for upon mutation of this residue to an asparagine, specificity of the enzyme was changed from GTP to xanthine triphosphate (XTP). Indeed, the mutant was determined to be more catalytically efficient utilizing XTP than the wild-type enzyme was with GTP (42). The importance of an aspartate in the GTP base recognition has been seen in other GTP binding enzymes, for example the elongation factor EfTu (43). The phosphate-binding loop was shown to contain the catalytic acid Asp^{13}, which when mutated to alanine yields a catalytically dead enzyme (44).

One characteristic of the G-protein superfamily is the 'P-loop' involved in binding the phosphates of the GTP (45). Containing the conserved 'P-loop', AMPSase is peripherally a member of the G-protein superfamily. For the members of the superfamily, GTP binding is a 'on/off' switch, where the unbound or GTP bound state of the protein is 'on', and hydrolysis to GDP turns the protein 'off' (45). Thus, GTP binding and subsequent hydrolysis to GDP is involved in the transduction of a signal within a cell for these proteins. In these proteins, the preference is to maintain the GTP bound state, and hydrolysis by the enzyme is slow. AMPSase is only peripherally a member of this superfamily as it utilizes the binding and hydrolysis of GTP to catalyze a reaction, instead of as an 'on/off' signal.

Two residues in the IMP binding site, Glu^{224} and Glu^{34}, yielded unique mechanistic implications. The side-chains of Glu^{224} and Glu^{34} are located in the vicinity of C6 of IMP, and thus postulated to be important in catalysis or substrate binding. Mutation of these residues to methinonine (Glu^{224}) or glutamine (Glu^{224} and Glu^{34}) has large effects on the catalysis of the enzyme (decreases k_{cat} 4- to 60-fold), but only minor effects on the kinetic parameters. The kinetic studies show that Gln^{224} works in concert with the catalytic acid,

Asp^{13}, to stabilize the 6-oxyanion form of IMP, allowing for nucleophillic attack on the γ-phosphate of GTP (46).

Determination of the critical interactions between the enzyme and the substrate L-aspartate are less well defined, as only hadacidin, an inhibitor competitive to L-aspartate, has been crystallized. The enzyme has shown the strictest specificity for substrates at this site, the only known substitute being hydroxylamine (8). The only previously identified residues important for aspartate recognition and binding consist of a group of conserved arginine residues (47). Mutation of Arg^{303}, Arg^{304} and Arg^{305}, individually, to Leu raised the K_m^{ASP} 50- to 200-fold, compared to wild-type AMPSase, while causing only minor decreases in k_{cat}. Indeed, arginine residues have been found interacting with the α- and β- carboxyl groups of aspartate in most aspartate binding proteins, for both enzymes and receptors (48-50).

Kang determined there are two Mg^{2+} ions required for activity in AMPSase. The first Mg^{2+} is associated with the β- and γ- phosphoryl groups of GTP, and Asp^{13} from the enzyme as seen in the crystal structure (37). The second Mg^{2+} Kang postulated to be coordinated in association with the α- and β- carboxyl groups and amino group of L-aspartate. The purpose of this association is to activate the protonated amino group, generating a better nucleophile for attack of the 6-phosphoryl IMP intermediate (51). Further support for the interaction of the α- and β-carboxyl group of aspartate with Mg^{2+} is that the alternative substrate hydroxylamine has a Hill coefficient of 1 (47). As of yet, a second Mg^{2+} ion has not been found in any crystal structure.

The first indication that the dimeric form of the enzyme is the active form are from a sub-unit complementation study, where two inactive mutants (Asp^{13}→Ala and Arg^{143}→Leu)

were combined and allowed to form a heterodimer. The heterodimer yields a single, competent active site per dimer (52). However, the complementation study cannot rule out the possibility of an active monomer for the wild-type enzyme.

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CHAPTER II: A STUDY OF *Escherichia coli* ADENYLOSUCCINATE SYNTHETASE ASSOCIATION STATES AND THE INTERFACE RESIDUES OF THE HOMODIMER*

A paper published in the Journal of Biological Chemistry†

Wenyan Wang‡, Andrea Gorrell‡, Richard B. Honzatko‡, and Herbert J. Fromm‡,#

ABSTRACT

The state of aggregation of adenylosuccninate synthetase from *Escherichia coli* is a point of controversy, with crystal structures indicating a dimer and some solution studies indicating a monomer. Crystal structures implicate Arg^{143} and Asp^{231} in stabilizing the dimer, with Arg^{143} interacting directly with bound IMP of the twofold related subunit. Residue $Arg^{143}$was changed to Lys and Leu, and residue Asp^{231} was changed to Ala. MALDI spectroscopy and analytical ultracentrifugation of the wild-type and the mutant enzymes indicate a mixture of monomers and dimers, with a majority of the enzyme in the monomeric state. In the presence of active site ligands, the wild-type enzyme exists almost exclusively as

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‡ Graduate students and professors, respectively. Gorrell performed mutagenesis, kinetic characterizaion and analytical ultracentrifugation of Arg^{143}→Lys.

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a dimer while the mutant enzymes show only slightly decreased dissociation constants for the dimerization. Initial rate kinetic studies of the wild-type and mutant enzymes show similar k_{cat} and K_m values for aspartate. However, increases in the K_m values of GTP and IMP are observed for the mutant. Changes in dissociation constants for IMP are comparable to changes in K_m values. Our results suggest that IMP binding induces enzyme dimerization, and that two residues in the interface region, Arg^{143} and Asp^{231}, play significant roles in IMP and GTP binding.

INTRODUCTION

Adenylosuccinate synthetase (AMPSase)[1] [IMP:L-aspartate ligase (GDP forming), EC 6.3.4.4.] catalyzes the first committed step in the conversion of IMP to AMP in the *de novo* synthetic pathway for purine nucleotides:

$$\text{IMP} + \text{L-aspartate} + \text{GTP} \xrightleftharpoons{Mg^{2+}} \text{adenylosuccinate} + \text{GDP} + P_i$$

Adenylosuccinate is then cleaved by adenylosuccinate lyase to form AMP and fumarate. AMPSase also plays a role in the salvage and nucleotide interconversion pathways (1). The enzyme is potentially a target of a natural herbicide (2) and of a drug used in the treatment of pediatric leukemia (3). Better understanding of the synthetase could support efforts in the design of drugs against HIV and cancer (4).

Three different mechanisms have been proposed for the catalytic action of AMPSase (1). The most widely accepted mechanism involves a 6-phosphoryl-IMP intermediate formed

[1] The abbreviations used are: AMPSase, adenylosuccinate synthetase; MALDI matrix-assisted laser desorption ionization.

by the nucleophilic attack of the 6-oxo group of IMP on the γ-phosphorus atom of GTP (5,6). A second nucleophilic attack by the amino group of aspartate on C6 of 6-phosphoryl IMP displaces the phosphate and forms adenylosuccinate.

AMPSase was first purified to homogeneity in 1976 from *Escherichia coli* (7) and has since been purified and characterized from many sources (1). The *purA* gene, which in *E. coli* codes for AMPSase, was cloned in 1986 (8) and used in the construction of an overexpression system (9). The crystal structure of AMPSase was determined to 2.8 Å (10) and later refined to 2.5Å and 2.0 Å (11). In crystal structures, the enzyme exists as a homodimer. Two nearly independent regions contribute to the interface between the polypeptide chains of the synthetase dimer. Residues putatively involved in the binding of IMP lie at or near the interface between polypeptide chains in the dimeric form of the enzyme. One of the residues, Asp^{231}, may play an important role in holding the subunits in close contact by hydrogen bonding to Arg^{147} and Lys^{140} of the 2-fold related subunit. Indeed, when Lys^{140} and Arg^{147} were replaced by isoleucine and leucine, respectively, $K_m^{(GTP)}$ and $K_m^{(IMP)}$ showed significant increases (12,13).

Crystal structures imply that Arg^{143} is involved in IMP binding to the active site of the symmetry-related subunit of the dimer. Arg^{143} may also stabilize the interface through at least one hydrogen bond. Slow phase inactivation of AMPSase was observed when guanosine-5'-O-[S-(4-bromo-2,3-dioxobutyl)] thiophosphate modifies the enzyme at Arg143. Modification of Arg143 is prevented by adenylosuccinate alone or by a mixture of GTP, $MgCl_2$ and IMP (14).

The cited findings suggest a direct relationship between dimer formation and AMPSase activity. AMPSase exists as a monomer in solution (15), yet the enzyme is a dimer

in crystal structures (10,11). The discrepancy between the level of aggregation of the enzyme in solution and the crystal is the basis of an important question: Does AMPSase function as a monomer or as a dimer? MALDI mass spectroscopy, analytical ultracentrifugation, and initial rate kinetics were used to determine the state of aggregation and activity of wild-type AMPSase and several interface mutants. Arg^{143} was replaced with leucine to remove the guanidinium group and yet retain some of the hydrophobic attributes of the original side chain. Mutation of Arg^{143} to lysine retains the positive charge, but limits hydrogen bonding opportunities of the side chain at position 143. Asp^{231} was replaced with alanine so as to disrupt the Lys^{140}-Asp^{231} salt link observed in the crystalline dimer. Our findings indicate that the *E. coli* enzyme dimerizes in response to active site ligands and that, on the basis of sequence homology, dimerization may be a property common to all known adenylosuccinate synthetases, regardless of source.

EXPERIMENTAL PROCEDURES

Materials—GTP, IMP, L-aspartate, phenylmethylsulfonyl fluoride, and bovine serum albumin were obtained from Sigma. A site-directed mutagenesis kit was obtained from Amersham. Restriction enzymes were obtained from Promega. *E. coli* strain XL-1 blue was obtained from Stratagene. *E. coli* strain *purA*$^-$ H1238 was a gift from Dr. D. Bachman (Genetic Center, Yale University). Other reagents and chemicals used in the experiments were obtained from Sigma if not specified.

Site-directed mutagenesis—Recombinant DNA manipulation was performed by using standard procedures (16). The plasmid containing a 1.8-kb BamHI - HindIII fragment from

PMS204 ligated into PUC118 was used in the mutagenesis step. All mutagenic oligonucleotide primers used in the experiments were synthesized on a Bioresearch 8570EX automated DNA synthesizer at the DNA Facility at Iowa State University. Mutagenesis was performed according to the protocol provided by Amersham. The mutations were confirmed by DNA sequencing using the chain termination method (17) at the Iowa State University DNA Facility. The 1.8-kb *Bam HI* - *Hind*III fragment with the desired mutation was ligated back into PMS204 and transformed into XL-1 blue cells. The plasmids isolated from that strain were used to transform *E. coli* strain *purA*⁻ H1238, the strain from which AMPSase was purified.

Protein assay—Protein concentration was determined by the Bradford (18) method using bovine serum albumin as the standard. Concentrations reported here refer to monomers.

Purification of wild-type and mutant adenylosuccinate synthetase—The wild-type and mutant enzymes were purified as described elsewhere (9). The purity of the enzyme was checked by SDS-PAGE according to Laemmli (19). AMPSase activity was determined as described earlier (20).

Kinetic studies of wild-type and mutant AMPSase—The concentrations of stock solutions of nucleotides were based on their molar extinction coefficients at 253 nm for GTP and 248 nm for IMP. For each enzyme assay, the increase at 290 nm was recorded at 25□C. The enzyme assay solution contained 20 mM Hepes (pH 7.7) and 5 mM $MgCl_2$. When GTP was the variable substrate, the concentration of aspartate was fixed at 5 mM, and the IMP concentration was fixed at 450 μM for wild-type AMPSase and at 12 mM for the mutants. When IMP was the variable substrate, the concentration of GTP was fixed at 300 μM, and

aspartate was fixed at 5 mM. When aspartate was the variable substrate, the concentration of GTP was fixed at 300 μM, and the concentration of IMP at 450 μM for the wild-type AMPSase and 12 mM for the mutants.

To obtain the values of K_{ia} and K_{ib}, dissociation constants for IMP and GTP, respectively, a 5X5 matrix of substrates were used with each enzyme. The enzyme assay solution contained 20 mM HEPES (pH 7.7), 5 mM $MgCl_2$, and 5 mM Aspartate, and varying GTP and IMP concentrations. The kinetic data were fit to equation 1 using a program written in Minitab in place of the Omnitab program described by Siano et al. (21):

$$\frac{1}{v}=\frac{1}{V_m}\left[1+\frac{K_a}{A}+\frac{K_b}{B}+\frac{K_{ia}K_b}{AB}\right] \quad (Eq.1)$$

It was assumed that the kinetic mechanism for the mutant enzymes was the same as the rapid-equilibrium Random mechanism described for the wild-type enzyme (5,6) and thus $K_{ia}K_b = K_aK_{ib}$.

In Equation 1, v is the initial rate, V_m is the maximal velocity, A is the GTP concentration, B is the IMP concentration, K_a is the Michealis constant for GTP, K_b is the Michealis constant for IMP, and K_{ia} is the dissociation constant for IMP. K_{ib} is the dissociation constant for GTP.

Circular Dichroisim Spectrometry—Circular dichroisim spectra for the wild-type and mutant enzymes were acquired on a JASCO J700 spectropolarimeter equipped with a data processor. Samples, 100 μg/ml of enzyme, dialyzed against 5 mM KPi (pH 7.0), 1 mM EDTA, 1 mM β-mercaptoethanol, were placed in a 1 cm cuvette, and data points were collected in 0.1 nm increments. Each spectrum was corrected for background contributions

of the buffer and smoothed using the spectropolarimeter program. The data were analyzed by JASCO analysis software or by PSIPLOT.

MALDI mass spectroscopy—All of the enzymes were dialyzed against 5 mM KPi buffer (pH 7.0), 1 mM EDTA, and 1 mM β-mercaptoethanol. Protein concentration was adjusted to 1 mg/ml. Samples of 0.5-1.0 μl were loaded with 0.5 μl of freshly made 3,5-dimethoxy-4-hydroxy-cinnamic acid matrix. Bovine serum albumin was used as the internal calibration standard. Data were collected on a Finnigan LASERMAT 2000 MALDI-TOF mass analyzer in the Protein Facility of Iowa State University and were analyzed by the LASERMAT 2000 data processing software.

Analytical ultracentrifugation (sedimentation equilibrium) of wild-type and mutant AMPSase — Analytical ultracentrifugation experiments were performed using a Beckman Optima XL-A ultracentrifuge. The temperature of the rotor (AN-60 Ti) was set a 4°C. Rotor speeds were set at 10K, 14K, and 18K. Wild-type and mutant AMPSase samples were prepared in 5 mM KPi buffer (pH 7.0) at concentrations of 2.9 to 11.6 μM corresponding to absorbences of 0.2 - 0.8 (280 nm, 1 cm cuvette). Concentration dependent equilibrium sedimentation was performed with concentrations of 4.4 μM and 58 μM, corresponding to A_{280nm} readings of 0.3 and 4.0. Samples of AMPSase in the presence of ligands were prepared by dialysis overnight against 5 mM KPi (pH 7.0), 5 mM succinate, 5 mM $MgCl_2$, 20 μM IMP, and 20 μM GTP. Protein samples were centrifuged at least 10 h before the collection of data. Stepwise radial scans were performed at 280 nm for the wild-type and mutant enzymes with and without ligands, and at 280 nm, 295 nm and 300 nm for the concentration dependent equilibrium sedimentation. Each reading is the average of 30 points, with nominal spacing of 0.001 cm between radial positions. Absorbence readings were

measured at 1 h intervals to ensure that equilibrium had been reached. Three scans were averaged and the data were analyzed by the method of Van Hold and Weischet (22) using the "IDEAL" model on the Optima XL-A Analysis Software (Version 2.0) to get the apparent molecular weight (MW_{app}). The partial specific volume of AMPSase, 0.737 cm^3/g, was calculated by the method of Cohn and Edsall (23).

Equilibrium sedimentation data for the wild-type and mutant enzymes obtained at three different rotor speeds were analyzed using 'SELF' model in multiple data set analysis program Optima XLA. The association constants (K_a) for the monomer-dimer equilibrium were obtained using the following equation:

$$c_r = E + c_{m,ro} \exp [M(1- \bar{\upsilon}\rho)\omega^2(r^2-r_o^2)/2RT] +$$

$$(c_{m,ro})^2 K_a \exp[2M(1- \bar{\upsilon}\rho) \omega^2(r^2-r_o^2)/2RT] \qquad \text{(Eq. 2)}$$

Where c_r is the concentration of the protein at a given radial position, $c_{m,ro}$ is the concentration of monomer at a reference position, M is the monomer molecular weight, $\bar{\upsilon}$ is the partial specific volume, ρ is the solvent density, ω is the angular velocity, r is the radial position in centimeters from the center of rotation, r_o is the distance in centimeters from the center of rotation to the meniscus, T is the absolute temperature, R is the gas constant, and E is a correction term for a non-zero baseline. The conversion of K_a into the dissociation constant K_d (μM) is performed as follows:

$$K_d = 2/\varepsilon l K_a = 24.2/K_a \ (\mu M) \qquad \text{(Eq. 3)}$$

where $\varepsilon = 68750\ M^{-1}cm^{-1}$ is the extinction coefficient for AMPSase at 280 nm and $l = 1.2$ cm is the path length of the centrifuge cells.

RESULTS

X-ray crystallographic studies suggest that interface residues play an important role in maintaining the quaternary structures of AMPSase (10,11). Two very important residues in this context are Arg^{143} and Asp^{231}. Arg^{143} in one subunit is ligated to the 5'-phosphoryl of IMP in the active site of the juxtaposed subunit (11). Asp^{231} forms a salt link with Lys^{140}, a residue putatively essential for AMPSase activity as a means of stabilizing subunit-subunit association. Experiments involving mutation of residues Arg^{143} and Asp^{231} were undertaken to gain insight into the role of these interface residues.

Comparison of the sequence 138-148 and 228-235 in *E. coli* AMPSase with the synthetases from other sources— Arg^{143} and Asp^{231} are conserved in the nine known AMPSase sequences (8, 24-29, 31)[2] (Table I), as are Arg^{147} and Lys^{140}. In crystal structures, the side chain of Asp^{231} forms a salt bridge with Lys^{140}, and the carbonyl of Asp^{231} hydrogen bonds to Arg^{147} (10,11). Chemical modification or mutation of Lys^{140} or Arg^{147} inactivates the synthetase (12,13). Arg^{143} from each subunit projects into the IMP binding site of the symmetry-related subunit of crystallographic dimers (11).

Mutagenesis of the E. coli AMPSase purA gene and purification of the mutant enzymes—The oligonucleotide primers used in the mutagenesis experiments are shown in Table II together with the sequencing primers for confirming the mutants. Arg^{143} was changed to leucine and lysine. Asp^{231} was mutated to alanine. These mutations altered the charge states of residues and/or hydrogen bonding interactions observed in crystallographic structures while keeping the size of the side chain comparable to that of the original residue. All the mutants were purified by using procedures similar to those for wild-type AMPSase,

with some modifications. The D231A mutant bound to the phenyl sepharose CL-4B so tightly that it could be eluted only by water. All the enzymes exhibited greater than 95% purity on the basis of SDS-PAGE and a molecular mass of 48-kDa (data not shown).

Secondary structure analysis— Circular dichroisim spectra of the mutant and wild-type enzymes were superimposable (data not shown) from 200 nm to 260 nm, indicating no global alterations in the secondary structures of the mutants relative to wild-type AMPSase.

Kinetic analysis of AMPSase mutants—The kinetic parameters for GTP, IMP, and aspartate with various forms of AMPSase are summarized in Table III. The K_m values for aspartate illustrate that the mutants differed little from that of the wild-type enzyme, suggesting that Arg^{143} and Asp^{231} are not involved in the binding of aspartate. In addition, the mutant and wild-type AMPSases had comparable k_{cat} values; however, K_m values for GTP and IMP exhibited significant increases for the mutant enzymes relative to wild-type AMPSase. The $K_m^{(GTP)}$ values for R143K, R143L, and D231A showed 2-, 10-, and 20-fold increases, respectively, compared with that of the wild-type enzyme. On the other hand, even more dramatic changes were observed for the K_m values of IMP. Increases in $K_m^{(IMP)}$ of 100-, 100-, and 60- fold for the mutants R143K, R143L and D231A, respectively, were observed relative to that of wild-type AMPSase. In addition, increases in $K_{ia}^{(IMP)}$ of 20- to 30-fold were also observed for the mutant enzymes, relative to that of the wild-type enzyme. These findings suggest that the residues in question contribute significantly to the binding of both IMP and GTP, either by stabilizing the dimer through hydrogen bonding, by direct interaction with IMP, or by both. The similar kinetic properties of R143K and R143L mutants with respect to IMP binding suggest that the positive charge of lysine alone did not

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[2] F. Tatum and M. A. Steckelberg, unpublished data

restore wild-type properties, suggesting a precise hydrogen bonding and stereochemical role for R143. The models for R143K and R143L are presented and discussed below (Fig. 1).

MALDI mass spectrometry analysis of AMPSase— AMPSase in solution is a monomer (15), whereas it is a dimer in crystals (10,11). We assumed that subunit association might involve interface residues such as Arg^{143}; therefore, experiments were undertaken to evaluate this possibility. The mutant enzymes and wild-type AMPSase were analyzed by MALDI mass spectroscopy. A typical spectrum is shown in Fig. 2. Given the estimated molecular mass of the monomer as 46 kDa, three ionized species were observed from 20-kDa to 110-kDa, corresponding to mass values of 23.4-kDa (M = 46.6-kDa, Z = 2), 46.6-kDa (M = 46.6-kDa, Z = 1), 92.7-kDa (2M = 92.7-kDa, Z = 1). The existence of a 92.7-kDa peak indicates the presence of dimers.

Equilibrium Sedimentation of wild-type and mutant AMPSases— Subunit association of wild-type AMPSase was evaluated at three different concentrations (32). Considering the molecular mass of the monomeric and dimeric enzymes, three different centrifugation speeds were utilized (10,000, 14,000, 18,000). The molecular mass of AMPSase does not depend on either the centrifugation speed or the protein concentration under the conditions tested. Typical equilibrium sedimentation data are shown in Fig. 3*a*. Fig. 3*b* shows the relationship between wild-type protein concentration and MW_{app}. At low concentrations (A_{280nm}= 0.1), the MW_{app} = 45 kDa, which matches the result determined by MALDI mass spectroscopy. At higher concentrations ($A_{280nm} \geq 0.65$), the MW_{app} approaches 60 kDa, which is between the molecular mass for monomeric and dimeric AMPSase. This observation strongly indicates the existence of a monomer-dimer equilibrium. Dissociation constants for dimer-monomer equilibria of wild-type and mutant AMPSases are shown in Table IV. At micromolar

concentrations of enzyme in the absence of ligands, the K_d values are approximately 10 μM for all the enzymes, indicating low concentrations of dimers under these conditions. In the presence of active site ligands the mutant enzymes exhibited small decreases in K_d values, suggesting a slight increase of dimers, whereas the wild-type enzyme exhibited a large decrease in K_d, implying that virtually all the protein is present as dimer. Active site ligands apparently are less effective in the stabilization of the mutant dimers. Our findings support the important roles played by the interface residues in forming AMPSase dimers.

DISCUSSION

Conflicting reports have characterized *E. coli* AMPSase as a monomer (15), and a dimer (9) in solution. Crystal structures of AMPSase clearly show the enzyme as a dimer in both the unligated (10, 11) and substrate-ligated states (33). AMPSase is putatively regulated by such feedback inhibitors as GDP, adenylosuccinate, and adenine nucleotides (1). The concentration of the latter class of compounds may not vary in the cell because of the adenylate kinase equilibrium. An alternative mode of regulation may be linked to the state of association of the enzyme. We therefore undertook experiments to determine the enzyme's state of association under different experimental conditions.

Protein aggregation is influenced by protein concentration, ligands, pH, temperature, and ionic strength (34-36). Discrepancies in reported molecular masses of AMPSase from different sources may stem from the precise conditions under which mass determinations were made (1). We used two widely differing approaches, MALDI mass spectroscopy and equilibrium sedimentation, to determine molecular mass. Data from MALDI mass spectroscopy clearly revealed the presence of a 92-kDa species (Fig. 2), a finding fully

consistent with the existence of AMPSase as a dimer in solution. The MALDI technique, however, cannot provide a measure of the relative amounts of monomer and dimer. Equilibrium sedimentation was performed to determine whether a dynamic equilibrium exists between the monomer and dimer forms. Our results suggest that without ligands, AMPSase is a mixture of both monomers and dimers, with the equilibrium greatly favoring the monomer - 70% monomer and 30% dimer at 11.4 μM wild-type enzyme, and the equilibrium shifts to favor the dimer at higher concentrations - 91% dimer and 9% monomer at 58.2 μM wild-type enzyme. These observations are in harmony with X-ray diffraction studies in which only dimers were observed (10, 11, 33).

We failed to observe two distinct species in centrifugation experiments, suggesting that a rapid equilibrium exists relative to the sedimentation rate between the monomers and dimers in the absence of ligands. Only dimers were detected, however, when substrates were added to the wild-type enzyme. Ligands clearly shift the equilibrium toward the dimer and the kinetic barrier in dimer formation and dissociation is relatively low. On the other hand, mutant enzymes in the presence of ligands exhibited only slightly lower K_d values than in the absence of ligands, indicating a much weaker ligand-induced dimerization of mutant enzymes relative to the wild-type enzyme. Considering that Arg^{143} and Asp^{231} are well conserved in all the AMPSase sequences, the results here also suggest that AMPSase from *E. coli* may require both subunits for catalytic activity at physiological ligand concentrations and that this may be a general property of all AMPSases, regardless of source. AMPSase, however is not the only protein that changes its state of subunit association upon ligand binding. Briehl demonstrated that lamprey hemoglobin exists predominantly as monomers when oxygenated and as oligomers when deoxygenated (37).

This study focused on two residues of AMPSase, Asp^{231} and Arg143, which are involved in putative subunit-subunit interactions. Arg143 from one subunit hydrogen bonds to the 5'-phosphoryl group of IMP in the active site of the symmetry-related subunit (33). In addition, Arg^{143} hydrogen bonds to a backbone carbonyl of the juxtaposed subunit. Because Arg^{143} is conserved in all nine known sequences of AMPSase (8, 24-29,31)[2], we suggest that its role in the *E. coli* enzyme is also conserved in all other known AMPSases. The mutants R143L and R143K exhibit approximately the same k_{cat} and K_m values for aspartate as does wild-type AMPSase with small changes in the K_m values for GTP (2- and 10- fold increase, respectively, for R143K and R143L). However, 100- fold increases in K_m for IMP were observed for both mutant enzymes. Despite great differences in the hydrophobicity and electrostatic charge of lysine and leucine residues at position 143, they exhibit very similar kinetic properties in terms of IMP binding.

The spatial relationship of the side chain of position 143 to the active site of the monomer related by the twofold symmetry is shown in Fig. 1. The guanidinum group of Arg^{143} bonds to the backbone carbonyl of the symmetry related subunit, as well as the 5'-phosphate of IMP (Ref. 33 and Fig. 1*b*). By model building, atom NZ of lysine 143 can occupy the same position as the guanidinum nitrogen responsible for the intersubunit hydrogen bonds (Fig. 1*c*). Thus, the increase in K_m for the R143K mutant is not immediately obvious from the modeling study. However, the substitution of a lysyl for an arginyl side-chain at position 143 leaves a packing void at the interface between monomers. Presumably this void is filled by a water molecule, in which case more than one conformational arrangement between that water molecule and the lysyl side chain is possible, but only one of

these arrangements is comparable to the interaction exhibited by Arg^{143} in the crystal structures.

Asp^{231}, also conserved in known AMPSase sequences, forms an intersubunit salt bridge with the side chain of Lys^{140} and a hydrogen bond to Arg^{147} through its backbone carbonyl. As for the R143K and R143L mutants, D231A showed about the same k_{cat} and K_m for aspartate as wild-type AMPSase. However, K_m values for GTP and IMP increased 20- and 60- fold, respectively, relative to the wild-type enzyme. These results are qualitatively similar to those for the R143K and R143L mutants and are consistent with the hypothesis that the dimeric form of the synthetase is functionally active (11). The increase in K_m for GTP with the mutants may be due to synergism in the binding of IMP and GTP. Markham and Reed, for instance, observed synergism in the inhibition of AMPSase by GDP and nitrate (38,39), both of which are competitive inhibitors with respect to GTP. Furthermore, on the basis of isotopic scrambling reactions (40), the γ-phosphoryl group of GTP probably exchanges between the two sites, where it is either covalently linked to the 6-oxo group of IMP or covalently linked to the β-phosphoryl group of the guanine nucleotide. In the absence of the dimer interface, AMPSase cannot provide an appropriate environment for binding IMP, which probably impairs the exchange process just described and leads to a weaker association of GTP with the enzyme.

Combining the two lines of evidence from biophysical analysis and initial rate kinetics on both the wild-type and mutant enzymes, we established a clearer relationship between the association states and enzymatic activity of *E. coli* AMPSase. MALDI mass spectroscopy revealed the existence of a 92-kDa species for all the mutant enzymes, indicating that mutations at positions 231 and 143 do not prevent dimerization. R143L, in

the presence of ligands, showed an apparent molecular weight of a dimer as determined by gel filtration analysis (data not shown). These observations suggest that neither Arg^{143} nor Asp^{231} are essential for dimer formation. On the other hand, position 231 and 143 mutants studied here exhibit little change in K_d values for the dimer to monomer equilibrium in the presence or absence of ligands, and higher Km(IMP) and Km(GTP) values with little change in k_{cat} values, implying that dimerization of AMPSase is required for the precise recognition and binding of substrates IMP and GTP. Assuming that the mutant enzymes retain the rapid equilibrium random ter ter mechanism of wild-type AMPSase (1), the comparable k_{cat} values for the mutants and wild-type AMPSase suggest neither Arg^{143} nor Asp^{231} play a role in the rate-limiting step of catalysis (interconversion from AMPSase-$MgGTP^{2-}$-IMP-aspartate to AMPSase-adenylosuccinate-$MgGDP^{1-}$-P_i).

An interesting question concerns the significance of the AMPSase monomer-dimer equilibrium and its physiological implications, if any. The enzyme should exist primarily as a monomer in the μM concentration range and substrates should shift the monomer-dimer equilibrium to dimer. Only if the monomer and dimer have dissimilar activities at physiological ligand concentrations can the monomer-dimer equilibrium be of significance to regulation. Interestingly, in the enzyme concentration range of 20 - 150 pM, there is a several minute lag in adenylosuccinate production when the reaction is initiated with the enzyme (data not shown). When the enzyme is preincubated with $MgGTP^{-2}$ and IMP and the reaction initiated with L-aspartate, however, the lag is eliminated. These findings are consistent with either an inactive monomer or a monomer of low activity relative to the dimer. Perhaps AMPSase is a monomer in the absence of nucleotide substrates, but is activated by *de novo*

purine nucleotide biosynthesis. This scenario may represent a major control mechanism of adenylosuccinate biosynthesis.

It is also noteworthy that AMPSase from *Saccharomyces cerevisiae* was identified as a single stranded DNA binding protein that specifically recognizes an autonomously replicating sequence (30). Because most of the single stranded DNA-binding proteins are oligomers, the dimerization phenomenon may indicate a dual role for AMPSase as an enzyme and as a regulatory element in DNA replication.

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Table I

Alignment of *E. coli* AMPSase amino acid sequence 138-148, 228-233 with the sequences of the AMPSase from other sources

Sources	Sequences*		References
	138-148	228-235	
Bacillus substilis	MD**K**AA**R**IGI**R**I	LDI**D**QGTY	24
Dictyostelium dicoidium	SS**K**AS**R**GGL**R**V	LDL**D**FGCY	25
Escherichia coli	ED**K**VA**R**RGL**R**V	LDI**D**HGTY	26
Human liver	SS**K**AA**R**SGL**R**M	LDI**D**FGTY	27
Mouse muscle	SS**K**AA**R**TGL**R**I	LDI**D**FGTY	28
Mouse T-lymphoma cells	SS**K**AA**R**SGL**R**M	LDI**D**FGTY	29
Haemophilus Influenzae	ED**K**VA**R**RGL**R**V	LDI**D**HGTY	30
Brucella abortus	ED**K**VG**R**RAI**R**V	LDN**D**HGTY	31
Saccharomyces cerevisiae	ST**K**AS**R**SGL**R**V	LDI**D**FGTY	32

*The conserved residues coresponding to K140, R143, R147 and D231 in *E. coli* AMPSase from different sources are shown in bold character.

Table II

Oligonucleotides used in site-directed mutagenesis and sequencing

Mutants	Sequences of primers*
R143L	CAGACCGCGAAGTGCTACTTTATC
R143K	CAGACCGCGTTTTGCTACTTTAT
D231A	TAAGTACCGTGGGCGATATCCAG
Sequencing primer for Residue 143:	CTGTGTGAAGCATGTCC
Sequencing primer for Residue 231:	ATGGCTGTTGCCGACATC

*Underlined letters indicate the mismatches

Table III

Kinetic parameters of wild-type and mutant AMPSases from *E. coli*[a]

Protein	k_{cat} (s^{-1})	$K_m^{(GTP)}$ (μM)	$K_m^{(IMP)}$ (mM)	$K_m^{(ASP)}$ (mM)	$K_{ia}^{(GTP)}$ (μM)	$K_{ia}^{(IMP)}$ (μM)
Wild-type	1.00 ± 0.05	26.2 ± 2.30	0.03 ± 0.00	0.23 ± 0.04	38.8 ± 6.40	74.1 ± 14.4
R143K	1.04 ± 0.01	58.3 ± 6.30	2.50 ± 0.25	0.26 ± 0.05	31.0 ± 5.92	1.50 ± 0.34
R143L	1.01 ± 0.01	283 ± 26.2	2.79 ± 0.13	0.34 ± 0.06	196 ± 37.6	1.26 ± 0.25
D231A	0.76 ± 0.02	503 ± 41.3	1.31 ± 0.23	0.50 ± 0.03	197 ± 62.3	20.4 ± 0.67

[a]Experimental conditions as described in Experimental Procedures.

Table IV

Monomer-Dimer Dissociation Constants

Enzyme	A_{280nm}	K_d[a] (A_{280nm})	K_d[b](μM)	Goodness of Fit[c]
In the absence of ligands				
Wild-type	0.3	0.464	11.2	0.030
	4.0	0.170	4.12	3.54
R143K	0.3	0.452	10.9	0.777
R143L	0.2	0.394	9.53	0.849
D231A	0.3	0.362	8.76	0.207
In the presence of ligands				
Wild-type	0.4	4.26×10^{-40}	1.03×10^{-38}	0.00019
R143K	0.4	0.178	4.31	1.96
R143L	0.4	0.307	7.43	2.07
D231A	0.4	0.208	5.03	0.151

[a]The dissociation constant in 280 nm absorbance units
[b]The dissociation constant in μM determined by equation (3) shown in "Experimental procedures."
[c]Goodness of fit was determined by the χ^2test.

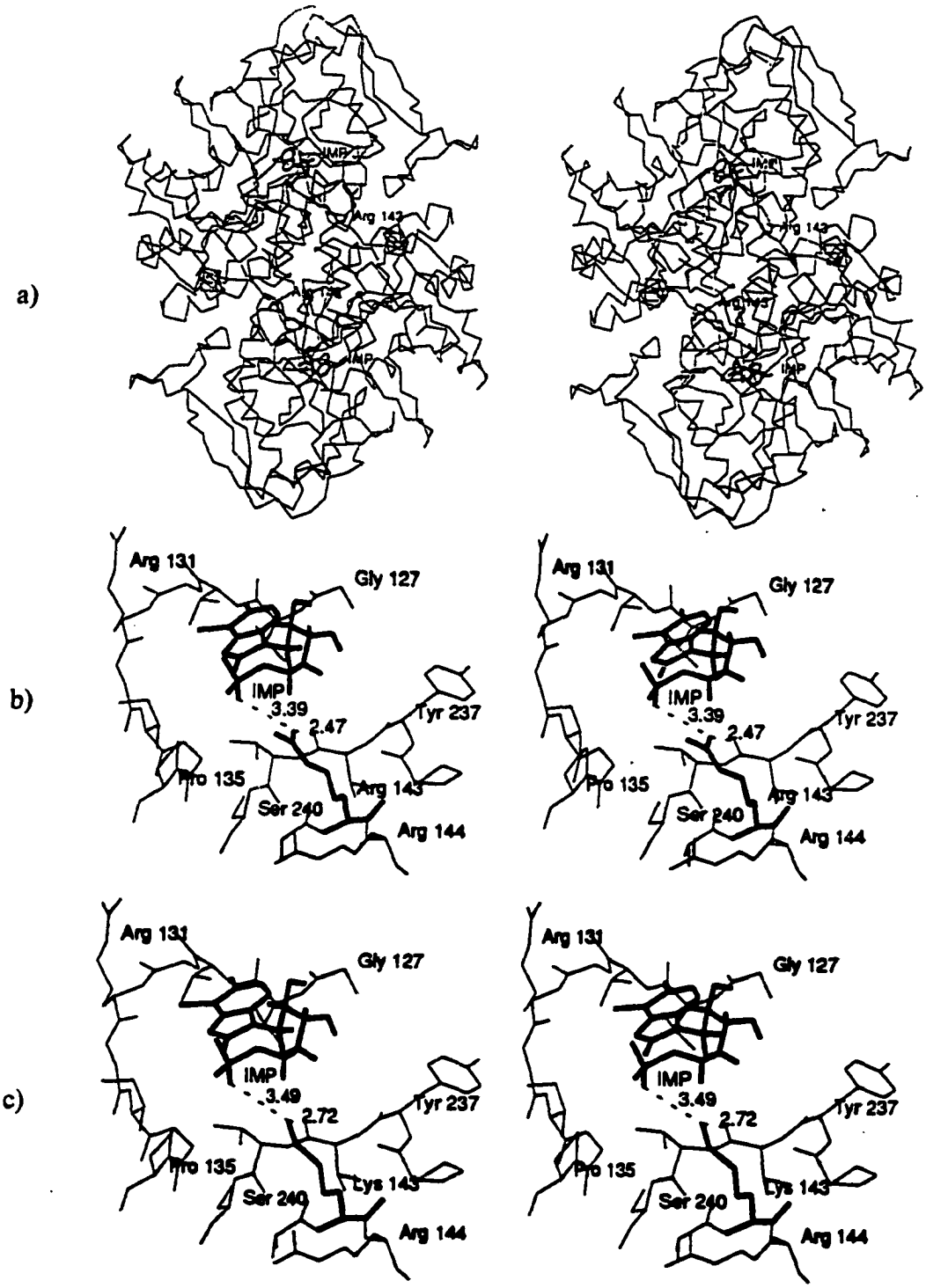

Fig. 1 a) Stereoview of the AMPSase dimer complexed with IMP

b) Stereoview of the interaction of IMP with Arg143

c) Stereoview of the interaction of IMP with modeled Lys143

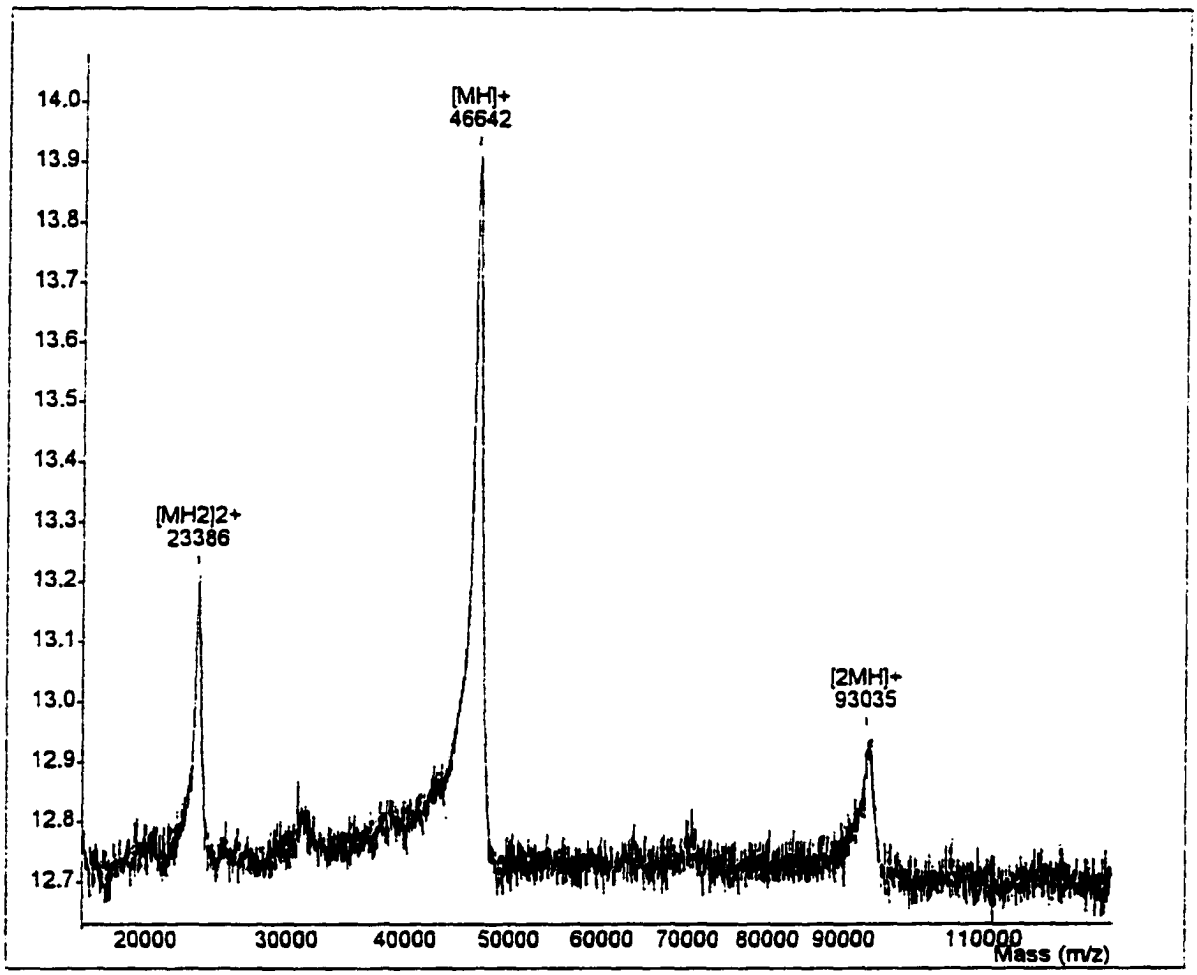

Fig. 2 Typical MALDI mass spectrum of wild-type and mutant enzymes of AMPSase. The $[MH2]^{2+}$ peak at 23383, $[MH]^{+}$ peak at 46634, and $[2MH]^{+}$ peak at 92693.

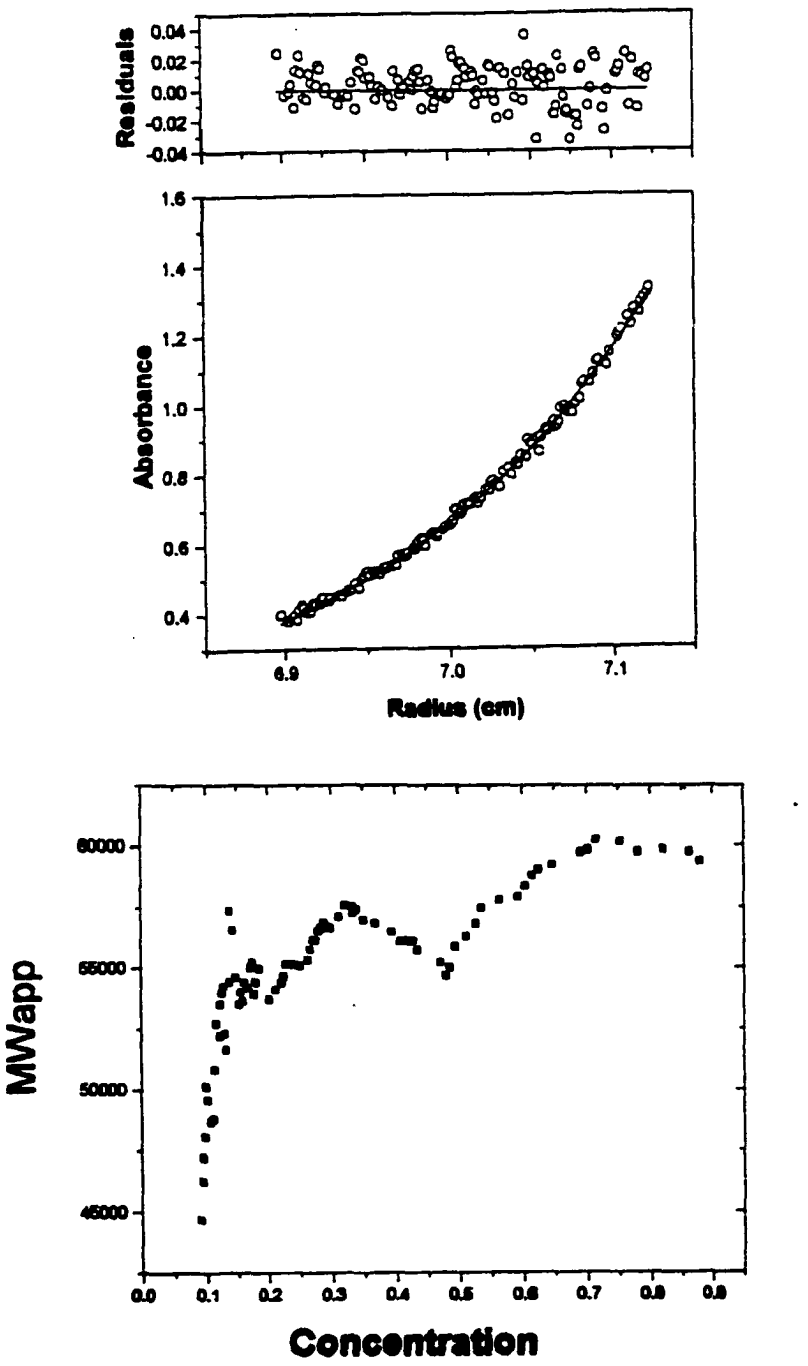

Fig. 3 a) Typical sedimentation equilibrium distribution of AMPSase in the absence of ligands.b) Apparent molecular mass of AMPSase as a function of protein concentration (in A_{280nm}) by the following equation: apparent molecular mass (in Da) = $(\delta(\ln c)/\delta r^2)$ x $(2RT/((1- \bar{\upsilon}\rho)\omega^2)$. *c*, AMPSase concentration shown as units of absorbance at 280 nm; *r*, radial distance from center of rotor; *T*, temperature in Kelvin; $\bar{\upsilon}$, partial specific volume; ρ, solution density; ω, angular velocity

CHAPTER III. AMBIGUITIES IN MAPPING THE ACTIVE SITE OF A CONFORMATIONALLY DYNAMIC ENZYME BY DIRECTED MUTATION: ROLE OF DYNAMICS IN STRUCTURE-FUNCTION CORRELATION IN *ESCHERICHIA COLI* ADENYLOSUCCINATE SYNTHETASE[*]

A paper published in the Journal of Biological Chemistry[1]

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ABSTRACT

On the basis of ligated crystal structures, Asn^{21}, Asn^{38}, Thr^{42}, and Arg^{419} are not involved in the chemical mechanism of adenylosuccinate synthetase from *Escherichia coli*, yet these residues are well conserved across species. Purified mutants (Asp^{21}→Ala, Asn^{38}→Ala, Asn^{38}→Asp, Asn^{38}→Glu, Thr^{42}→Ala and Arg^{419}→Leu) were studied by kinetics, circular dichroism spectroscopy and equilibrium ultracentrifugation. Asp^{21} and Arg^{419} are not part of the active site, yet mutations at positions 21 and 419 lower k_{cat} 20-fold

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[†] Three graduate students and professors, respectively. Paper written by Wang and Gorrell with assistance from Honzatko and Fromm. Mutations Asn^{38}→Asp and Arg^{419}→Leu, pH kinetics and ultracentrifugation of Asn^{38}→Asp performed by Gorrell.

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and 10-fold, respectively. Thr^{42} interacts only through its backbone amide with the guanine nucleotide, yet its mutation to alanine significantly increases K_m for all substrates. Asn^{38} hydrogen bonds directly to the 5'-phosphoryl group of IMP, yet its mutation to alanine and glutamate has no effect on K_m values, but reduces k_{cat} by 100-fold. The mutation Asn^{38}→Asp causes 10- to 57-fold increases in K_m for all substrates along with a 30-fold decrease in k_{cat}. At pH 5.6, however, the Asn^{38}→Asp mutant is more active, yet binds IMP 100-fold more weakly, than the wild-type enzyme. Proposed mechanisms of ligand induced conformational change and subunit aggregation can account for the properties of mutant enzymes reported here. The results underscore the difficulty of using directed mutations alone as a means of mapping the active site of an enzyme.

INTRODUCTION

Adenylosuccinate synthetase (IMP:L-aspartate ligase (GDP forming) E.C. 6.3.4.4, AMPSase[2]) is an essential enzyme in most organisms (for review, see reference 1), catalyzing the first committed step in the biosynthesis of AMP:

IMP + L-aspartate + GTP ⇔ adenylosuccinate + GDP + phosphate

Primary sequences of AMPSase (2-10) are 40% identical for any pairwise comparison, indicating a strong tendency to preserve a primordial gene throughout evolution (10). The enzyme putatively facilitates the formation of 6-phosphoryl-IMP by the nucleophilic attack of the 6-oxo group of IMP on γ-phosphate of GTP, and then the formation of adenylosuccinate by displacement of the 6-phosphoryl group by L-aspartate (11, 12).

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[2]Abbreviations used are: AMPSase, adenylosuccinate synthetase; ASP, L-aspartate; KP_i, potassium phosphate

AMPSase from *Escherichia coli* is a monomer at physiological concentrations (1 μM), but dimerizes when nucleotide ligands are present (13, 14). The dimer is probably the physiologically active form of the enzyme, having a K_m corresponding to intracellular concentrations of IMP.

The disordered active site of unligated AMPSase becomes ordered in the presence of substrates and substrate analogs (15-20). The largest conformational change is a 9 Å movement of the loop 42--53 (40s loop), which folds against the guanine nucleotide. Loop 120-131 (120s loop), which interacts with IMP, and loop 299-303 (300s loop), which interacts primarily with analogs of L-aspartate in crystal structures, become ordered in the presence of ligands. The conformational changes above putatively exemplify induced fit, a concept introduced by Koshland some three decades ago (21). The ligand-enzyme interactions, however, which contribute most (in terms of a thermodynamic driving force) to the observed conformational changes have yet to be identified, nor can we exclude the possibility of energy contributions from interactions between protein residues well-removed from the active site.

On the basis of ligated crystal structures of adenylosuccinate synthetase, Asp^{21}, Asn^{38}, Thr^{42} and Arg^{419} do not interact with atoms of substrates involved with the chemistry of phosphotransfer or nucleophilic attack by L-aspartate (17,19). In fact, Asp^{21} and Arg^{419} do not interact with ligands (16,17,19,20). The backbone amide of Thr^{42} forms a hydrogen bond with the α-phosphoryl group of the guanine nucleotide, but its side chain forms only a weak hydrogen bond to that same α-phosphoryl group (20). Asn^{38} provides one of 5 hydrogen bonds to the 5'-phosphoryl group of IMP; implicating Asn^{38} in the ground-state stabilization of the IMP-enzyme complex (17,19). Nevertheless, mutations of Asn^{38}, have little effect on

the K_m of IMP, but a major impact on k_{cat}, mutations of Asp^{21} and Arg^{419} destabilize the transition state, and the mutation of Thr^{42} to alanine reduces affinities for all substrates. The effects of each of the mutations above can be understood in terms of their influence upon two interdependent, dynamic mechanisms in AMPsase: (i) ligand induced reorganization of the active site and (ii) ligand induced dimerization of the enzyme. The above mutations also illustrate the importance of a sound understanding of structure and dynamics of an enzyme before assigning functional roles to side chains.

EXPERIMENTAL PROCEDURES

Materials— GTP, IMP, L-aspartate (ASP), phenylmethylsulfonyl fluoride, and bovine serum albumin came from Sigma. Restriction enzymes came from Promega. *Pfu* DNA polymerase and *E. coli* strain XL-1 blue were obtained from Stratagene. *E. coli* strain H1238 (*purA*$^-$) was a gift from Dr. D. Bachman (Genetic Center, Yale University). Phenyl Sepharose CL-4B came from Pharmacia. Other reagents and chemicals came from Sigma if not otherwise specified.

Overexpression of wild-type and mutant AMPSases— Due to the undetectable levels of expression for some mutants using the PMS204 plasmid expression system, we used here a more efficient prokaryotic expression system, based on the pTrc99A vector (Pharmacia).

The *purA* gene was amplified by PCR using the following primers to incorporate Nco I and Pst I restriction sites at either end of the *pur A* gene.

TrcN 5'-GATGCCATGGGTAACAACGTCGTCG-3' (*Nco*I site underlined)

TrcC2 5'-AACTGCAGTCTGCCAGGCGTACCACA-3' (*Pst*I site underlined)

The *NcoI* restriction site was incorporated into the N-terminus of the *purA* gene, before the first Met codon (ATG), and the *PstI* restriction site was introduced after the stop codon. *Pfu* DNA polymerase was used in the PCR reaction to ensure high-fidelity amplification (Fig 1).

The amplified and subcloned *purA* gene containing the 1.3 kb fragment was sequenced twice in both directions using the chain termination method (22) at the Nucleic Acid Facility, Iowa State University. Sequence analysis showed 100% identity with the *E. coli pur A* sequence deposited in GENBANK, except at position 415, a GAT (Asp) is replaced by GGT (Gly), which agrees with published crystal structures (15). The *pur A* gene was subcloned into the pTrc99A vector and the final construct, pTrpA, was transformed into *E. coli* strain H1238 (*purA*$^-$) for overexpression of AMPSase.

Site-directed mutagenesis— The *NcoI - PstI* fragment containing *pur A* was subcloned in to the pAlterEXII vector (Promega). Site-directed mutagenesis was carried out according to published procedures from Promega, Inc. The primers used in mutations were

5'-GTAAGATCGTCGCTCTTCTGACTGA-3' (Asp^{21}→Ala),

5'-GGGCGGTCACGCTGCAGGCCATA-3' (Asn^{38}→Ala),

5'-GGGCGGTCACGACGCAGGCCATA-3' (Asn^{38}→Asp),

5'-GGGCGGTCACGAAGCAGGCCATA-3' (Asn^{38}→Glu),

5'-CGCAGGCCATGCTCTCGTAATCAA-3' (Thr^{42}→Ala), and

5'-CCGGATCTTACTGAAACCATG-3' (Arg^{419}→Leu)

where the underlined bases are mismatched with respect to the wild-type *purA* sequence. The primer 5'-AACAACGTCGTCGTACTGGG-3' was used to sequence the plasmids and confirm mutations for all but Arg^{419}→Leu, where the primer 5'-

AACAGCCAAGCTTGCATGCC-3' was used. All primers were synthesized on a Bioresearch 8570EX automated DNA synthesizer at the Nucleic Acid Facility at Iowa State University. After mutagenesis, the *Nco*I - *Pst*I fragment containing the desired mutation was subcloned into pTrc99A and transformed into the *E. coli* strain H1238 (*pur A*$^-$) for expression.

Preparation and kinetics of wild-type and mutant AMPSases— The wild-type and mutant enzymes were purified as described elsewhere (23-25) with the following modifications. The enzyme was eluted from a phenyl sepharose CL-4B column by a series of buffered solutions, 0.6 M, 0.4 M and 0.2 M in $(NH_4)_2SO_4$ and 50 mM in potassium phosphate (KP_i) (pH 7.0). All the enzymes eluted at 0.2 M $(NH_4)_2SO_4$. The enzyme fractions were concentrated and dialyzed against 50 mM KP_i (pH 7.0), then further purified using a DEAE-TSK HPLC column. Enzyme purity was monitored by SDS-PAGE (26), and protein concentrations were determined using Bradford reagent (Bio-Rad) (27). All the mutant enzymes were subjected to circular dichorism analysis as described elsewhere (22-24). From 1-500 μg/mL enzyme was used in kinetic assays, depending on the activity of each mutant. Absorbance changes at 290 nm and 25°C were monitored with a GBC model 918 UV/visible spectrophotometer equipped with a Peltier-Effert temperature controller.

pH effects on AMPSase activity— MES and HEPES were chosen as buffers for the pH range 5.5 - 8.5, and acetate buffer was used at pH 5.2. Buffer concentrations were held at 20 mM for all assay solutions. Assay solutions contained 300 μM GTP, 5 mM $MgCl_2$, 5 mM ASP for the wild-type enzyme and 1.5 mM GTP, 7 mM $MgCl_2$, 10 mM ASP for the $Asn^{38}\rightarrow Asp$ mutant enzyme. IMP concentrations varied from 25-750 μM for assays of the wild-type

enzyme, and 25 μM to 10 mM for assays of the Asn^{38}→Asp mutant. 500 ug/mL of the Asn^{38}→Asp mutant and 1 μg/mL of wild-type AMPSase were used in assays. 2 mm quartz cuvettes were used to compensate for the high absorbance due IMP. Longer assay time (4 min) was used to compensate for the low activity of the Asn^{38}→Asp mutant.

Analytic equilibrium sedimentation ultracentrifugation— Wild-type, Asn^{38}→Asp and Asn^{38}→Glu mutant AMPSases were analyzed by analytical equilibrium sedimentation ultracentrifugation, in the absence and in the presence of ligands (5 mM $MgCl_2$, 30 μM IMP, 30 μM GTP, 1 μM hadacidin) at pH 7.7 and 5.6. Equilibrium ultracentrifugation experiments were performed as described previously (13), using hadacidin in place of aspartate. Enzyme concentrations were such that they gave an OD_{280} reading of 0.4. Monomer-dimer dissociation constants were determined as previously described (13).

RESULTS

Sequence comparison and crystallographic analysis— Positions corresponding to Asp^{21}, Asn^{38}, His^{41}, Thr^{42} and Arg^{419} are almost 100% identical across species (Table I). In the absence of ligands Asn^{38} has no specific interactions, but in the ligated synthetase it hydrogen bonds with the 5'-phosphate of IMP [Fig. 2; (17)]. His^{41}, a putative catalytic acid (17,28), has three mutually exclusive sets of interactions: (i) In the unligated synthetase His^{41} hydrogen bonds with Asp^{21} (16, 17). (ii) In the complex of GDP, NO_3^-, IMP, Mg^{2+}, and hadacidin, His^{41} hydrogen bonds with the β-phosphoryl group of GDP (17). (iii) In the complex of GDP, 6-thiophosphoryl-IMP, Mg^{2+}, and hadacidin, His^{41}, hydrogen bonds with the 6-thiophosphoryl group and to Glu^{221} (19). Asp^{21} in the unligated synthetase hydrogen

bonds with His^{41}, as noted above, but also makes a salt link with Arg^{419} in ligated complexes (17). Thr^{42} has no specific interaction in the unligated synthetase (16, 17), but in ligated complexes its backbone amide interacts with the α-phosphoryl group of GDP and its backbone carbonyl hydrogen bonds with a water molecule, which in turn hydrogen bonds with the 2′-OH of GDP and backbone carbonyl 417. In one of three ligated complexes of the synthetase (17,19,20), the side chain of Thr^{42} may interact weakly with the α-phosphate of GDP [oxygen to oxygen, donor-acceptor distance of approximately 3.1 Å (20)]. The interactions above are summarized in Table II.

Expression, purification, and characterization of wild-type and mutant AMPSases— All proteins have an apparent monomer molecular mass of 48 kDa and purity greater than 95% as shown by SDS PAGE. pTrpA plasmids containing the mutations of this study complement the *purA*⁻ auxotroph on M9 minimal medium, supplemented with thronine and arginine, indicating sufficient enzyme activity to sustain the transformed cells.

Secondary structure analysis— The CD spectra of Asp^{21}→Ala, Thr^{42}→Ala, Arg^{419}→Leu and wild-type enzymes are identical from 200 to 260 nm, indicating the absence of conformational change due to mutation. The position 38 mutants showed small differences in their CD spectra relative to the wild-type spectrum (data not shown). The differences are probably a consequence of a perturbation in the conformation of the 40s loop and/or the monomer-dimer equilibrium of AMPsase (see below).

Kinetic analysis of wild-type and mutant enzymes— The K_m values of the Asp^{21}→Ala, Asn^{38}→Ala, and Asn^{38}→Glu mutants are comparable to those of wild-type AMPSase (Table III). Although k_{cat} for the Thr^{42}→Ala mutant was the same as that of the wild-type enzyme,

K_m values showed 5-to 10-fold increases. The mutation of Asp^{21} to alanine reduced k_{cat} 20-fold and slightly increased K_m^{GTP} and K_m^{IMP}, while Arg^{419} reduced k_{cat} 10-fold, with a slight increase in K_m^{ASP}, and 8-fold increases in K_m^{GTP} and K_m^{IMP}. Asn^{38}→Ala, Asn^{38}→Asp and Asn^{38}→Glu mutants showed 30- to 200-fold reductions in k_{cat} relative to the wild-type enzyme. Of the three position Asn^{38} mutants, only Asn^{38}→Asp exhibited increased K_m values (K_m^{IMP} increased 80-fold).

pH dependent kinetic studies of wild-type and Asn^{38}→Asp— Of the three position 38 mutants, only K_m values for the Asn^{38}→Asp mutant show large increases. Altered kinetic parameters for the Asn^{38}→Asp mutant could originate from electrostatic repulsion between the 5'-phosphoryl group of IMP and the side chain of Asp^{38}. Protonation of the Asp^{38} side chain, or the 5'-phosphoryl group of IMP, then, could restore k_{cat} and K_m to wild-type levels. k_{cat} versus pH profiles for the wild-type and the Asn^{38}→Asp enzymes are bell-shaped (Fig. 3). The optimum pH for wild-type and Asn^{38}→Asp enzymes is 7.8 and 5.6, respectively. In fact, the Asn^{38}→Asp mutant had higher catalytic activity than the wild-type enzyme at pH 5.6 (Table III) and 5.2 (data not shown). Although wild-type levels of activity were recovered in the Asn^{38}→Asp mutant by dropping the pH, K_m^{IMP} remains 100-fold higher than that of the wild-type enzyme.

Analytic equilibrium ultracentrufugation— IMP induces dimerization of AMPsase monomers (13,14). Furthermore, the dimer arguably has 100-fold higher affintiy for IMP than the monomer. Hence, we examined the Asn^{38}→Asp mutant by ultracentrifugation methods, in order to determine whether its elevated K_m^{IMP} originated from its properties of

aggregation. In centrifugation studies reported here, the wild-type and Asn^{38}→Glu enzymes are controls, as each exhibits comparable K_m values for IMP, and hence should be dimers in the presence of IMP. The monomer-dimer dissociation constants for the three enzymes in the absence and presence of ligands at pH 7.7 are in Table IV. At pH 5.6, the three enzymes precipitated during ultracentrifugation runs. The three AMPSases show similar K_D values in the absence of ligands. In the presence of ligands, however, the K_D of the Asn^{38}→Asp mutant (252 μM) differs significantly from that of wild type and Asn^{38}→Glu enzymes, both of which are vanishingly small (Table IV). Furthermore, the dissociation constant for Asn^{38}→Asp in the presence of ligands is 19 times higher than the dissociation constant for Asn^{38}→Asp in the absence of ligands.

DISCUSSION

For the rapid-equilibrium mechanism of AMPSase (1), k_{cat} represents the breakdown of the quarternary complex of enzyme and substrates to enzyme and products. Thus, k_{cat} for AMPsase is sensitive to the energy changes in the transition state. Kinetic data indicate destabilization of the transition state for the Asp^{21}→Ala and Arg^{419}→Leu mutants (k_{cat} falls 20- and 10-fold, respectively, relative to the wild-type enzyme). Thus, the Asp^{21}-Arg^{419} salt-link must stabilize the transition state. As the hydrogen bond between Asp^{21} and Arg^{419} is about 13 Å from the bound Mg^{2+} (approximate center of catalysis), the stabilization is necessarily due to an indirect mechanism. The loss of the Asp^{21}-Arg^{419} salt-link could destabilize interactions between the 40s- and 400s-loop. Indeed, in the unligated enzyme Arg^{419} is disordered (15, 16) and the 40s- and 400s-loop do not interact. In the ligated

enzyme backbone carbonyl 417 hydrogen bonds with a water molecule, which in turn hydrogen bonds to backbone carbonyl 42 and the 2′-OH of GDP (17).

An alternative mechanism by which the loss of the Asp^{21}-Arg^{419} salt-link could influence the transition state is by way of a small perturbation of the P-loop (residues 8-16). Asp^{13} is a putative catalytic base (17,28). A displacement of its side chain by perhaps as little as 0.5 Å could lead to complete inactivation of the synthetase. The interaction of Asp^{21} with Arg^{419} could stabilize the position of helix H1, which lies on the C-terminal side of the P-loop. Mutations in the P-loop have only modest effects on K_m^{GTP} and no effect on the K_m for other substrates (28), consistent with the lack of change in K_m values for the $Asp^{21}\rightarrow$Ala mutant.

The mutation of Arg^{419} to leucine has a minor effect on the K_m values for IMP and GTP. The increased K_m^{GTP} must be due to a localized change in the 400's loop as the Asp^{21} mutation has no effect on K_m values. The mutation of Arg^{419} could perturb the stacking of Pro^{417} with the guanine base and hence lead to the increase in K_m^{GTP}. Given the observed binding synergism of IMP and GTP (13), a reduced GTP interaction should weaken IMP binding.

The side chain of Thr^{42} binds weakly (at best) to the α-phosphoryl group of GTP, yet it is a conserved residue and its mutation to alanine increases K_m values by approximately 10-fold. The above phenomenon may be a consequence of the 40s-loop, which is in an equilibrium between two conformational states. The "open" conformer predominates in the absence of ligands, whereas the "closed" conformer appears when ligands are bound to the active site. The absence of an appreciable effect on k_{cat} due to the mutation of Thr^{42} to alanine implies no significant perturbation of the 40s-loop in the ligated conformation of the

synthetase. If however, the mutation to alanine stabilizes the "open" conformation of the loop, then ligands must spend a greater fraction of their binding energy to drive the 40s-loop to its "closed" conformation. That the K_m values increase for all substrates is a reflection of synergism in substrate binding (13).

Asn^{38}→Ala and Asn^{38}→Glu have no influence on K_m values but reduce k_{cat} by 200- and 30-fold, respectively. Evidently, the interaction between Asn^{38} and the 5'-phosphate of IMP does not enhance the affintiy of IMP for the active site. Instead the binding energy probably is diverted completely to the stabilization of the transition state. Asn^{38} belongs to a short element (residues 38-41) which immediately precedes the 40s-loop. Within this structural element, the peptide link between residues 40 and 41 undergoes a significant change in conformation (17) and the α-carbon and side-chain of Asn^{38} move toward the 5'-phosphate by 1 Å. These small conformational adjustments preceding the 40s-loop are probably coupled to the 9 Å movement of the loop itself. Hence the energy of interaction of Asn^{38} with the 5'-phosphate of IMP, may go to support the conformational change in the 40s-loop.

The mutation of Asn^{38} to aspartate presents a far more complex phenomenon. The introduction of a negative charge at position 38 probably elevates K_m^{IMP} by charge repulsion. All of the allowable conformations of Asp^{38} bring its side chain close to the 5'-phosphate of IMP. (In contrast, allowable conformations of Glu^{38} are possible which bring about significant separation of the 5'-phosphate and the side chain). By protonation of Asp^{38} and/or the 5'-phosphate of IMP, a hydrogen bond is possible in the Asn^{38}→Asp mutant. Thus, k_{cat}

for the mutant at pH 5.6 is restored to wild type levels, possibly because IMP can again drive the required conformational change in the 40s-loop.

K_m values for the Asn^{38}→Asp mutant are not restored to wild-type levels at low pH however, suggesting that a pH-independent mechanism is responsible for the elevated K_m values of the mutant. The wild-type enzyme is predominantly a monomer in the absence of ligands at concentrations used in assays (13). Furthermore, IMP induces dimerization of the wild-type enzyme at pH 7.7; its interaction with Arg^{143} of a second monomer being essential in stabilizing the dimer. The mutation of Arg^{143} to leucine or lysine does not influence k_{cat}, but increases K_m^{IMP} 100-fold at pH 7.7, and concomitantly abolishes ligand-induced dimerization at low concentrations of IMP and enzyme (13). The high K_m^{IMP} values for the Asn^{38}→Asp mutant at pH 7.7 then may reflect the weak interaction of IMP with the mutant as a monomer, rather than the binding of IMP to a mutant dimer. This is supported by the monomer-dimer dissociation constants for Asn^{38}→Asp with and without ligands. The higher dissociation constant for the Asn^{38}→Asp in the presence relative to the absence of ligands shows that Asn^{38}→Asp favors a dimeric state in the absence of ligands. At pH 5.6 IMP may bind to the active site of the mutant as a monoanion, allowing formation of the hydrogen bond with the side chain of Asp^{38}. However, the monoanionic state of IMP may not stabilize sufficiently its interaction with Arg^{143} of a second subunit. Hence, the affinity for IMP remains low, because the mutant remains a monomer at low pH. The wild-type enzyme, on the other hand, selects the dianion state of IMP from solution at low pH and, as a consequence, dimerizes. Hence, the K_m^{IMP} for wild-type enzyme at pH 5.6 reflects the interaction of IMP with a synthetase dimer.

AMPsase is rife with ligand-induced changes, which in a broad perspective influence subunit dimerization and/or reorganization of its active site. As a consequence of the coupling of ligand-binding energy to dynamic processes which influence the conformation of loci remote from the active site, residues can bind directly to substrates and have no effect on binding affinity as measured by K_m and residues remote from the active site can have substantial effects on the stability of the transition state or the binding affinity of substrates. Such phenomena underscore the pitfalls in using site directed mutations alone in assigning residues to the active site of an enzyme.

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Table III

Kinetic parameters of wild-type and mutant AMPSases

AMPSase	pH	k_{cat} (s^{-1})	K_mGTP (μM)	K_mIMP (μM)	K_mASP (mM)	k_{cat}/K_mGTP (s^{-1} μM^{-1} x10^{-3})	k_{cat}/K_mIMP (s^{-1} μM^{-1} x10^{-3})	k_{cat}/K_mASP (s^{-1} mM^{-1})
wild-type	7.7	1.00±0.05	26±2	28±1	0.23±0.04	38±4	36±3	4.4±0.8
	5.6	0.096±0.008	11±1	70±20	0.9±0.2	9±1	1.4±0.4	0.11±0.03
Asp^{21}→Ala	7.7	0.049±0.001	52±5	48±4	0.17±0.01	0.95±0.09	1.02±0.08	0.30±0.02
Arg^{419}→Leu	7.7	0.100±0.002	250±20	200±10	0.34±0.07	0.41±0.03	0.49±0.03	0.29±0.06
Asn^{38}→Ala	7.7	0.0048±0.0002	41±5	49±7	0.30±0.02	0.12±0.02	0.10±0.01	0.016±0.001
Asn^{38}→Asp	7.7	0.0134±0.0003	270±20	1600±400	2.6±0.4	0.051±0.004	0.008±0.002	0.0052±0.0007
	5.6	0.171±0.008	116±10	3800±400	0.5±0.1	1.5±0.2	0.045±.002	0.33±0.09
Asn^{38}→Glu	7.7	0.034±0.001	54±5	28±1	0.24±0.02	0.62±0.06	1.22±0.06	0.143±0.01
His^{41}→Asn[a]	7.7	0.0095±0.0001	130±10	400±8	2.0±0.6	0.076±0.008	0.024±0.002	0.005±0.002
Thr^{42}→Ala	7.7	0.890±0.02	280±20	123±5	1.4±0.1	3.2±0.2	7.2±0.3	0.65±0.05

[a]From Kang, C., Sun, N., Poland, B. W., Gorrell, A., Honzatko, R. B., and Fromm, H. J. (1997) *J. Biol. Chem.* 272, 11881 - 11885

Table IV

Monomer-dimer dissociation constants for wild-type and mutant AMPSases

	Absence of Ligands		Presence of Ligands	
AMPSase	K_D (μM)	Goodness of fit[a]	K_D (μM)	Goodness of fit[a]
Wild-type	15 ± 2	0.0009	0 ± 0.00006	0.2287
$Asn^{38} \rightarrow Asp$	14 ± 2	0.6014	250 ± 70	1.130
$Asn^{38} \rightarrow Glu$	24 ± 4	2.187	0 ± 0.0005	3.278

[a]Goodness of fit determined by the chi-square test (29)

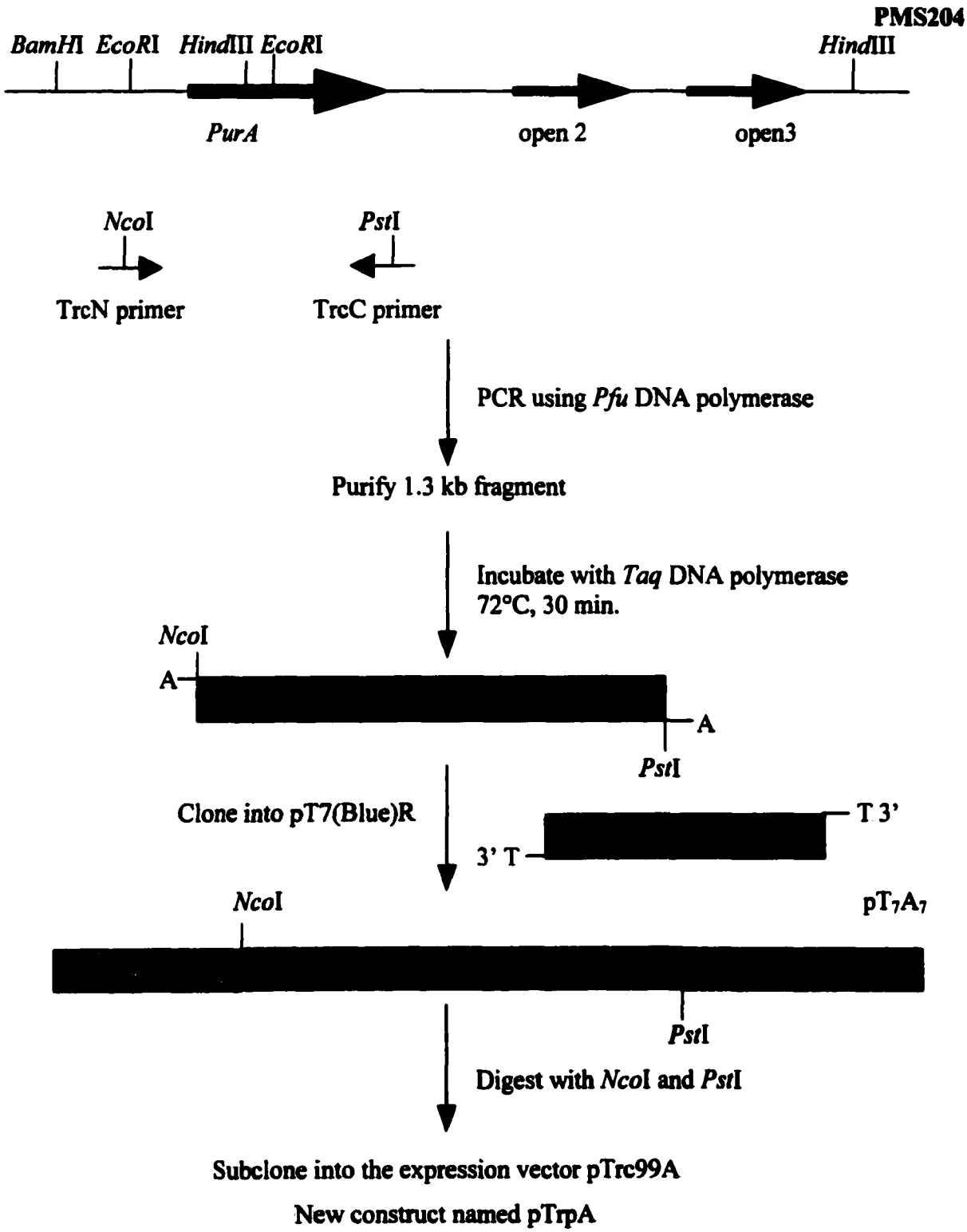

Figure 1. Consruction of vector pTrpA for overexpression of AMPSase.

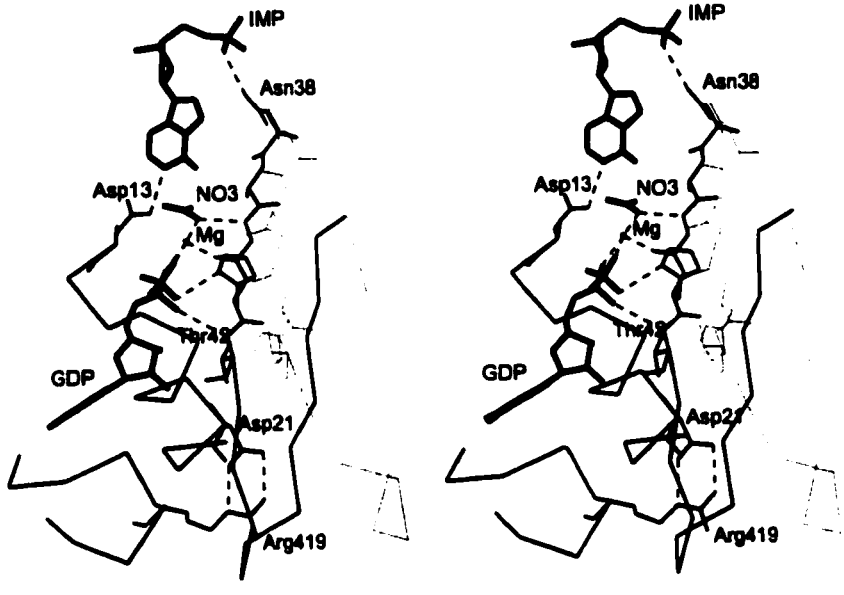

Figure 2. Stereoview of conformational changes in the active site of AMPsase.

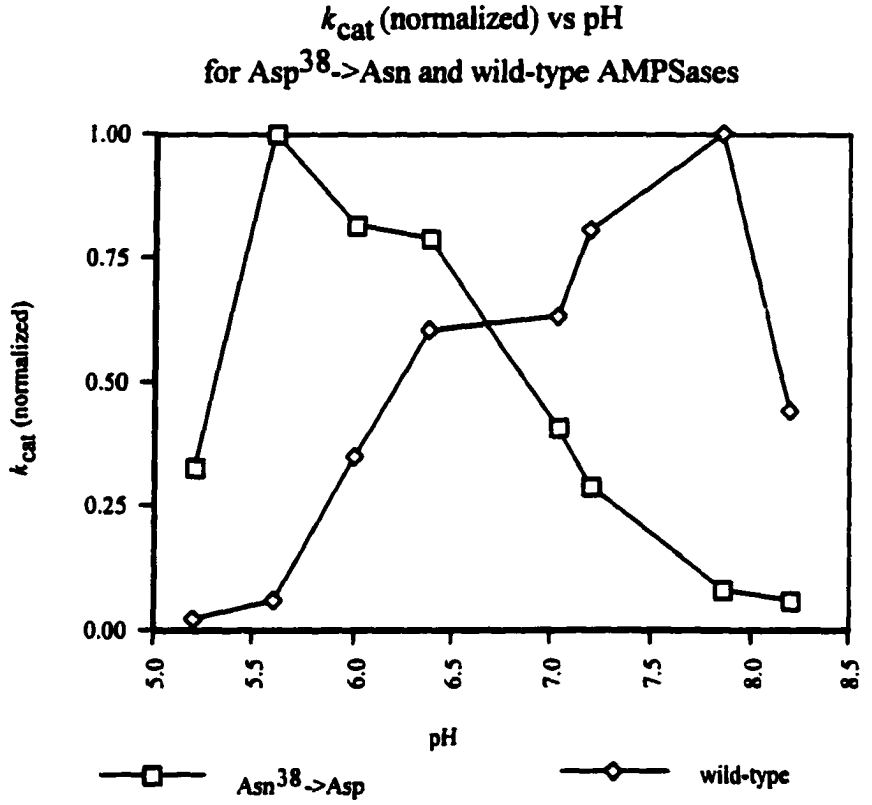

Figure 3. k_{cat} versus pH profiles of wild-type and Asn^{38}→Asp mutant AMPsase. The k_{cat} values for both enzymes were normalized for clarity of comparison. The maximum k_{cat} value is 1.59 s^{-1} for the wild-type enzyme at pH 7.8, and 0.237 s^{-1} for Asn^{38}→Asp mutant enzyme at pH 5.6.

CHAPTER IV. DIRECT AND INDIRECT DETERMINANTS OF SUBSTRATE RECOGNITION IN *ESCHERICHIA COLI* ADENYLOSUCCINATE SYNTHETASE*

A paper prepared for the Journal of Biological Chemistry

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ABSTRACT

A collection of β-branched amino acids in the active site of adenylosuccinate synthetase from *Escherichia coli* exhibit a remarkable variation as to the degree and mechanism by which they influence substrate recognition and catalysis. Perturbations in the steady-state kinetics caused by directed mutations of Thr^{128}, Thr^{129}, Val^{273}, Thr^{300}, and Thr^{301}, reflect the stabilization of enzyme-substrate complexes by direct hydrogen bonding, by limitation of conformational flexibility in the protein, and by limiting nonproductive conformations of bound substrates. Thr^{129} and Thr^{301} hydrogen bond directly to IMP and L-aspartate (ASP), respectively. Mutations of each residue to alanine either completely

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† Three graduate students and professors, respectively. Paper written by Gorrell and Wang with the assistance of Honzatko and Fromm. Mutations Thr^{128}Ala, Thr^{300}Val, and all Val^{273} mutants prepared and kinetics performed by Gorrell.

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inactivate the synthetase (Thr^{301}→Ala) or increase the K_m for IMP by 30-fold. Mutation of Thr^{300} to alanine increases the K_m for ASP by 30-fold, but the effect is indirect, as the mutation of Thr^{300} to valine reduces the K_m for ASP by only 3-fold, and yet increases k_{cat} by 300% relative to wild-type enzyme. Similarly, the mutation of Thr^{128} to alanine increases the K_m for IMP by 10-fold due to a perturbation in hydrogen bonding between Thr^{129} and IMP. Val^{273} can interact with ASP only through non-bonded contacts, yet its mutation to alanine, threonine or asparagine causes 35-, 15- and 40-fold increases in the K_m of ASP. Inhibition of the wild-type and position-273 mutant enzymes by succinate, fumarate and maleate is consistent with Val^{273} acting sterically to block nonproductive complexes of dicarboxylic acids with the active site. Factors which maintain the strict conservation of Val^{273} across all known adenylosuccinate synthetases are discussed.

INTRODUCTION

In a broad range of organisms and cell-types, adenylosuccinate synthetase (IMP:L-aspartate ligase (GDP forming) E.C. 6.3.4.4, AMPSase[1]) plays a significant role in *de novo* purine synthesis, the purine nucleotide cycle, and/or salvage pathways for nucleotides (for reviews see (1-3)). AMPSase converts IMP and L-aspartate (ASP) to adenylosuccinate using GTP as an energy source. The synthetase from *Escherichia coli* is a homodimer (4). IMP may play an important physiological role in the regulation of synthetase activity by stabilizing the assembly of inactive monomer into active dimers (2,5,6). As indicated by investigations, first in kinetics (7-11) and then in X-ray crystallography (12-14), the catalytic mechanism

[1] Abbreviations used: AMPSase, adenylosuccinate synthetase; CD, circular dichroism; ASP, L-aspartate.

involves a nucleophilic attack on the γ-phosphoryl group of GTP by the 6-oxo group of IMP to form a 6-phosphoryl intermediate of IMP. The displacement of that 6-phosphoryl group by the amino group of ASP generates adenylosuccinate. The synthetase enforces strict determinants for substrate specificity; only 2'-deoxy-IMP and β-D-arabinosyl-IMP substitute for IMP, 2'-deoxy-GTP and GTPγ(S) for GTP, and DL-threo-β-fluoroaspartate, alanine-3-nitronate, cysteine sulfinate, L-alanosine, and hydroxylamine for ASP (7,15-17).

Three loops, residues 40—52, 120—130, and 298—304, undergo conformational transitions in response to active site ligands (13,18-20). Directed mutations of residues in or near Loop 40—52 have generated unanticipated results. Asn^{38} in all ligated crystal structures of the synthetase, for instance, hydrogen bonds with the 5'-phosphoryl group of IMP. Yet, the mutation of Asn^{38} to alanine has no effect on the K_m for IMP, but reduces k_{cat} by three orders of magnitude (21). Evidently, the energy associated with the Asn^{38}-IMP interaction goes entirely toward the stabilization of the transition state. Hence, a crystal structure by itself does not reveal the extent to which interactions stabilize the transition state as opposed to the ground state of enzyme-substrate complexes.

Recent crystal structures and/or the sequence conservation amongst adenylosuccinate synthetases implicate Thr^{128}, Thr^{129}, Val^{273}, Thr^{300}, and Thr^{301} in important functional roles. All of the above residues except Val^{273} belong to flexible loops, which undergo significant conformational change in response to the binding of IMP (Loop 120—130) or ASP (Loop 298—304). Val^{273} is conserved in all known sequences of AMPSase, yet on the basis of crystal structures, has no obvious role in structural stability or in the recognition of substrate. Directed mutation, steady-state kinetics and circular dichroism (CD) spectroscopy reveal the

extent to which these residues contribute to the stability of the transition state and/or to the binding affinity of substrates. We report here a remarkable variation as to the degree and mechanism by which this set of β-branched residues influence substrate recognition and catalysis by adenylosuccinate synthetase.

EXPERIMENTAL PROCEDURES

Materials— Restriction enzymes came from Promega. *Pfu* DNA polymerase and *E. coli* strain XL-1 blue were obtained from Stratagene. *E. coli* strain H1238 (*purA*⁻) was a gift from Dr. D. Bachman (Genetic Center, Yale University). Phenyl Sepharose CL-4B came from Pharmacia, and hydroxylamine hydrochloride from Fisher Scientific. Other reagents and chemicals came from Sigma, unless specified otherwise.

Expression of wild-type and mutant enzymes— Details regarding the construction of pTrpA are in the literature (21). The *pur A* gene in PMS204 was amplified using PCR and *Pfu* DNA polymerase, and subcloned into pTrc99A (Pharmacia). The resulting plasmid (pTrpA), was transformed into *E. coli* strain H1238, which cannot express the endogenous synthetase (*purA*⁻).

Directed mutations— The *NcoI*—*PstI* fragment containing *purA* was subcloned into pAlterEXII (Promega). Site directed mutagenesis followed the protocol provided by Promega, Inc. The following primers were used in directed mutations:

5'- AGCGATCGGCGCCACCGGTCG-3' for Thr^{128}→Ala,

5'- CGATCGGCACCGCCGGTCGTGGT -3' for Thr^{129}→Ala,

5'- ACTCCACTCGTGCAGGTGCAGGTC-3' for Val^{273}→Ala,

5'- ACTCCACTCGTACAGGTGCAGGTC-3' for Val^{273}→Thr,

5'- ACTCCACTCGTAATGGTGCAGGTC-3' for Val^{273}→Asn,

5'- GAATTCGGCGCAGCTACGGGGCG-3' for Thr^{300}→Ala, and

5'- TTCGGCGCAACTGCGGGGCGTCG-3' for Thr^{301}→Ala.

Nucleotide residues underlined above are mismatched with respect to the wild-type sequence. The sequencing primer used to confirm mutations at positions 128 and 129 was 5'-TCTGAAGCATGTCCGCTGATC-3', and that used for mutations at 273, 300 and 301 was 5'-CTGCTGGATATCGACCACGG-3'. Oligonuceotide syntheses and DNA sequences (dideoxy dye termination protocol) were done by the Nucleic Acid Facility, Iowa State University. The *NcoI*—*PstI* fragment with the desired mutation, was subcloned into pTrc99A, and transformed into *E. coli, pur A*$^-$ strain H1238 for expression.

Preparation of wild-type and mutant AMPSases— Wild-type and mutant enzymes were purified as described elsewhere (22-24) with the following modifications. The phenyl sepharose CL-4B column was washed in succession with solutions of 0.6, 0.4 and 0.2 M $(NH_4)_2SO_4$, buffered by 50 mM KP_i, pH 7.0. Wild-type and mutant synthetases eluted from the column in 0.2 M $(NH_4)_2SO_4$. Appropriate fractions were pooled, concentrated and dialyzed against 50 mM KP_i, pH 7.0, then further purified by DEAE-TSK HPLC chromatography (Toso-Haas). Enzyme purity was monitored by SDS-PAGE (25), and concentrations were determined by the Bradford method (26), using the reagent from Bio-Rad. CD spectra for mutant and wild-type synthetases were taken as described elsewhere(22-24).

Kinetic assays using L-aspartate— Kinetic parameters of wild-type and mutant AMPSases were determined at 25° C by monitoring absorbance changes at 290 nm with a GBC Scientific Equipment model 918 GBC UV/visible spectrophotometer, equipped with a Peltier-Effert temperature controller. Enzyme assay solutions contained 20 mM Hepes, pH 7.7, 5 mM $MgCl_2$, and fixed concentrations for two of the three substrates, as appropriate, in the determination of K_m for the remaining substrate (11). Fixed concentration of GTP, IMP and ASP were 0.30, 0.50 and 5.0 mM, respectively. 1—200 μg/mL of wild-type or mutant enzyme was used in each assay, depending upon its specific activity. Determination of k_{cat} and K_m values employed the program ENZFITTER (27).

Determination of K_i for inhibitors— The inhibition constants for succinate, fumarate, and maleate were determined utilizing a 5 X 5 matrix of substrate and inhibitor for each enzyme/inhibitor combination. Assay contained 20 mM HEPES, pH 7.7, 5 mM $MgCl_2$, 500 μM IMP, and 300 μM GTP. Enzyme concentrations were 4 μg/mL wild-type, 30 μg/mL Val^{273}→Ala, 20 mg/mL for Val^{273}→Asn, 66 mg/mL Val^{273}→Thr. Aspartate was varied from from 0.5 to 5X K_m (depending upon mutant examined) and inhibitor concentrations were 0 - 15 mM succinate, 0 - 16.7 mM fumerate, or 0 - 10 mM maleic acid. All inhibitors were determined to be competitive with respect to aspartate, and fitting to the equation for competitive inhibition

$$\frac{1}{v} = \frac{1}{v_{max}} \left[1 + \frac{K_a}{A} + \frac{K_{ia} I}{K_I A} \right] \qquad \text{(Eq. 1)}$$

was performed by linear regression analysis utilizing the MINITAB program (28). In Equation 1, v is the initial velocity, v_{max} is the maximal velocity, A is the ASP

concentration, I is the inhibitor concentration, K_a is the Michaelis constant for ASP, K_i is the dissociation constant for the inhibitor, and K_{ia} is the dissociation constant for ASP.

Kinetic assays using hydroxylamine (NH_2OH)— 3 M NH_2OH was freshly made from solid hydroxylamine hydrocholoride, and adjusted to pH 7.7 by the addition of potassium hydroxide. UV difference spectra of the reactions were recorded at 25° C on a GBC UV/VIS 918 spectraphotometer, equipped with a Peltier-Effert temperature controller. The reaction solution contained 20 mM Hepes (pH 7.7), 1 mM GTP, 450 μM IMP, 6 mM $MgCl_2$ and 400 mM NH_2OH , with 20 μg/mL of wild-type or mutant enzyme. The spectra obtained at time zero was subtracted from the spectra collected at subsequent time points (Fig. 1). The $\Delta\lambda_{max}$ in difference spectra occurred at 270, 264 and 270.5 nm, in the presence of ASP, ASP with excess adenylosuccinate lyase, and NH_2OH, respectively. Recorded spectra were consistent the formation of 6-N-hydroxy-AMP, when hydroxylamine was used in place of ASP (7). Kinetic assays were conducted at 290 nm, using 2 mm quartz cuvettes to offset strong absorbance due to GTP at 280 nm. At 290 nm the extinction coefficient the conversion of one mole of IMP into product is 3.51×10^6.

RESULTS

Conservation of target residues— Thr^{129} and Val^{273} are found in all 33 complete cDNA sequences of adenylosuccinate synthetases, whereas Thr^{128} and Thr^{301} are present in 32 of these sequences (serine was the alternative residue). In 24 of 32 sequences position 300 is threonine, the alternative residue-type being valine. Overall identity amongst sequences with the adenylosuccinate synthetase family is approximately 46%.

Purity and secondary structure of mutant enzymes— The mutant and wild-type enzymes migrate comparably on phenyl sepharose and DEAE columns. All proteins were at least 95% pure on the basis of SDS-PAGE. The CD spectra of all enzymes were identical from 200—260 nm (data not shown), indicating no major changes in secondary structure.

Kinetics of mutant enzymes— The K_m^{ASP} and k_{cat} for Thr^{128}→Ala and Thr^{129}→Ala are comparable to those of the wild-type enzyme (Table I). In contrast Thr^{128}→Ala exhibits 10- and 2-fold increases in K_m^{IMP} and K_m^{GTP} , respectively, relative to wild-type enzyme, and Thr^{129}→Ala exhibits 30- and 80-fold increases, respectively, in the same parameters. Mutations of position 273 cause minor changes in K_m^{GTP} and K_m^{IMP} , but K_m^{ASP} increases 32-, 40-, and 15-fold for the Ala^{273}, Asn^{273}, and Thr^{273} mutant enzymes, respectively. The k_{cat} for Val^{273}→Asn is 20-fold less than that of the wild-type enzyme, whereas k_{cat} for Val^{273}→Ala is triple that of the wild-type enzyme. The mutation of Thr^{300} to alanine elevates K_m^{GTP} by 5-fold and K_m^{ASP} by 30-fold. The K_m^{GTP} and K_m^{IMP} increase by approximately 2-fold or less due to the Thr^{300}→Val mutation, and k_{cat} and K_m^{ASP} increase 3-fold. The mutation of Thr^{301} to alanine results in the complete loss of activity under conditions employed here (20 mM Hepes, pH 7.7, 1 mM GTP, 500 μM IMP, 6 mM Mg^{2+}, 25 mM ASP, 200 μg/ml of protein).

Ala^{301}, Ala^{273}, Thr^{273}, Asn^{273} and wild-type AMPSases exhibit similar kinetic parameters when hydroxylamine replaces ASP (Table II). K_m^{NH2OH}, K_m^{GTP} and K_m^{IMP} vary over a 3-fold range, whereas k_{cat} varies 5-fold amongst the enzymes of Table II. The Hill coefficient for Mg^{2+}, however, falls from 2 to unity when NH_2OH replaces ASP (data not shown).

Inhibition of wild-type enzyme and position 273 mutant synthetase by succinate, fumarate and maleate varies over a broad range of affinities (Table III). All kinetic mechanisms of inhibition are competitive with respect to ASP, and hence probably represent ligand interactions at a common locus within the active site. K_i^{SUC} is at least 11-fold higher for wild-type and other position 273 mutants relative to the Thr^{273}. Fumarate is not an inhibitor of the wild-type synthetase, but exits fairly potent inhibition of the Asn^{273} mutant. On the other hand, maleate is 40-fold more potent as an inhibitor of the Thr^{273} mutant than the wild-type enzyme.

DISCUSSION

The residues studied here cluster in the vicinity of the ASP and IMP binding sites (14,20). The side-chain of Thr^{128} hydrogen bonds with Asp^{114}, as Thr^{129} hydrogen bonds with the 5'-phosphoryl group of IMP through its backbone amide group and its side chain. Interactions of threonines 128 and 129 putatively stabilize the IMP-bound conformation of the Loop 120—130, and enhance the affinity of the active site for IMP. The side-chain of Thr^{300} hydrogen bonds with backbone carbonyl 38 and the side-chain of His^{53}, but perhaps more importantly, backbone amide 300 hydrogen bonds with the carboxyl group of hadacidin, a competitive inhibitor of ASP (Fig. 2). The carboxyl group of hadacidin is putatively the structural analogue of the β-carboxyl group of ASP. Thr^{301} hydrogen bonds with the carboxyl group of hadacidin through its backbone amide and its side-chain, and also hydrogen bonds through its side-chain with Arg^{303}. Arg^{303} in turn also hydrogen bonds with the carboxyl group of hadacidin. The interactions of Thr^{301} may be important in the

recognition of ASP and in the stabilization of an intramolecular hydrogen bond between its β-carboxyl group bond and its α-amino group (12,20). The β-carboxyl group of ASP putatively abstracts a proton from the α-amino group ASP in the second-step of the synthetase reaction (nucleophilic attack of the α-amino group of ASP on the atom C-6 of 6-phosphoryl-IMP). The side-chain of Val^{273} makes nonbonded contacts with hadacidin. Although side-chains larger than the isopropyl group at position 273 could come into steric conflict with ASP, there are no obvious negative consequences in the substitution of side-chains of smaller or similar in size to the isopropyl group. The backbone carbonyl of Val^{273} hydrogen bonds with the 2'-hydroxyl group of IMP, but this interaction may not be important as 2'-deoxy-IMP is a good substrate for the synthetase (29).

Thr^{128}→Ala and Thr^{129}→Ala mutants exhibited 10- and 30-fold increases in K_m^{IMP}, respectively, while retaining wild-type levels of activity. Thus, Thr^{128} and Thr^{129} enhance the affinity of the active site for IMP. Presumably the hydrogen bond between Thr^{128} and the conserved Asp^{114} must stabilize the ligand bond conformation of Loop 120—130 , enhancing the interactions between Thr^{129} and the 5'-phosphoryl group of IMP. Evidently, only a small portion of the binding energy of the side-chain interactions of threonines 128 and 129 goes to the stabilization of the transition state (k_{cat} falls only twofold due to these mutations). In this regard, threonines 128 and 129 are similar to Arg^{143}. The side-chain of Arg^{143} from a symmetry related monomer of the synthetase dimer hydrogen bonds with the 5'-phosphoryl group of IMP. The mutation of Arg^{143} to leucine or lysine results in a 100-fold increase in K_m^{IMP} and no change in k_{cat} (6,30). In contrast, the mutation of Asn^{38}, which also hydrogen

bonds with the 5'-phosphoryl group of IMP, has no effect on K_m^{IMP}, but causes a 1000-fold decrease in k_{cat} (21).

The 60-fold increase in K_m^{GTP} relative to the wild-type enzyme for the Thr^{129}→Ala mutant must come from an indirect mechanism, as Thr^{129} and GTP are separated by more than 10 Å. A 60-fold increase in K_m^{GTP} was observed as well for Arg^{143}→Lys and Arg^{143}→Leu mutant enzymes (6,30). Correlated changes in K_m^{IMP} and K_m^{GTP} probably stem from the binding synergism between IMP and GTP, which may originate from the interaction of Asn^{38} with the 5'-phosphoryl group of IMP. In a crystal structure of an IMP-synthetase complex (Hou, Z., Fromm, H. J. and Honzatko, R. B., unpublished), Loop 40—52 adopts the conformation of the GDP-bound synthetase in the absence of guanine nucleotide. Evidently, IMP alone through its interaction with Asn^{38}, can stabilize the GDP-bound conformation of Loop 40—52. Correlated changes in K_m^{IMP} and K_m^{GTP} due to mutations at positions 129 and 143, may stem from a perturbation of the Asn^{38}-IMP interaction and a concomitant loss in the ability of IMP to drive conformational changes in Loop 40—52.

The significant effects on K_m^{ASP} and/or k_{cat} due to mutations of threonines 300 and 301 are in harmony with those observed in mutations of Arg^{303}, and Arg^{304} and Arg^{305} to leucine (31). Crystallographic structures implicate Loop 398—304 in the recognition of the β-carboxyl group of ASP (12,20). The 30-fold increase in K_m^{ASP} for the Thr^{300}→Ala mutant is probably due to weakened hydrogen bonds between the β-carboxyl group of ASP and backbone amide 300, backbone amide 301, and the side-chain of Thr^{301}. The mutation of Thr^{300} to alanine eliminates hydrogen bonds with backbone carbonyl 38 and His^{53}, interactions which evidently help Loop 298—304 in its ligand-bound conformation. Other,

more significant factors are at play here as well, however. The β-branched side-chain at position 300 limits allowable main-chain torsion angles relative to the methyl group of alanine. The Thr^{300}→Val mutant enzyme, which retains conformational restraints on the backbone, but eliminates side-chain hydrogen bonds, causes only a 3-fold increase in K_m^{ASP} and actually enhances k_{cat} by 300%. The modest effects of the Thr^{300}→Val mutation are consistent with the presence of valine at position 300, as an alternative to threonine, in 25% of known sequences for the synthetase. The 5-fold increase in K_m^{GTP} due to the mutation of Thr^{300} to alanine may reflect binding synergism between ASP and GTP. GTP/ASP binding synergism may stem in part from the coordination of each ligand to a common Mg^{2+}. Furthermore, in a series of crystal structures Choe *et al.* (14) demonstrate enhanced interactions between Arg^{305} (which putatively binds to the α-carboxyl group of ASP) and the α-phosphoryl group of the guanine nucleotide in the presence of hadacidin.

As Thr^{301}→Ala mutant is inactive using ASP in assays, we are unable to determine whether the loss of activity is due to an effect on k_{cat} or to a large increase in K_m^{ASP}. If the mutation of Thr^{301} to alanine eliminates critical hydrogen bonds with the β-carboxyl group of ASP, as inferred by crystallographic complexes (12,20), then the Thr^{301}→Ala mutant enzyme and the wild-type enzyme should respond comparably to hydroxylamine as an alternative substrate. Indeed, kinetic parameters of the Thr^{301}→Ala mutant are similar to those of the wild-type enzyme (Table III). As the K_m for hydroxylamine is 1000-fold higher than the K_m^{ASP} for the wild-type enzyme, the reaction involving hydroxylamine may be diffusion-controlled. Increased values for K_m^{GTP} and K_m^{IMP} when using hydroxylamine as a substrate may then reflect the loss of binding synergism between ASP and the nucleotides

The Hill coefficient for Mg^{2+} when the synthetase is assayed in the presence of ASP and hydroxylamine is 2 and 1, respectively. The reduced Hill coefficient in synthetase-mediated reactions which employ hydroxylamine reaffirms the correlated binding of ASP and a second Mg^{2+} in the kinetic mechanism of the synthetase (32). Evidently, only one Mg^{2+} participates in the hydroxylamine reaction, whereas two are involved in the ASP reaction. Kang and Fromm suggest that a second Mg^{2+} binds to the carboxyl groups of ASP, in order to reduce the pK_a of the α-amino group. An alternative model puts the second Mg^{2+} between the α-carboxyl group of ASP and the side chain of Asp^{13} (3). Both models are consistent with the reduced Hill coefficient observed in the presence of hydroxylamine.

Although only non-bonded contacts are evident in crystal structures between Val^{273} and hadacidin, the mutation of Val^{273} to alanine causes a staggering 35-fold increase in K_m^{ASP}. As the mutation puts a small side-chain in place of a larger one, increased steric interactions with the substrate are unlikely. Furthermore, the binding of ASP in its catalytically productive modes to the Ala^{273} mutant and wild-type enzymes must be nearly identical, as K_m^{GTP} (which reflects ASP/GTP-binding synergism) and k_{cat} are unchanged. Mutation to a larger side chain (Val^{273}→Asn) causes a 40-fold increase in K_m^{ASP} and a 20-fold reduction in k_{cat}, suggesting a perturbation in the mode of productive binding for ASP, due perhaps to the steric effects from a side chain with increased size. The isosteric mutation Val^{273}→Thr, however, still causes a 15-fold increase in K_m^{ASP}, while k_{cat} actually increases 200%.

Inhibition of position 273 mutants by dicarboxylic acids provides a measure of insight as to how Val^{273} functions in substrate recognition. Fumarate is a potent inhibitor of the

Ala^{273} and Asn^{273} mutant enzymes, but has no effect on the wild-type enzyme, and similarly succinate and maleate become potent inhibitors of the Thr^{273} mutant enzyme. The active sites in position 273 mutants generally recognize a more diverse set of dicarboxylic acids than does the wild-type active site. This broadened recognition of dicarboxylic acids, may allow ASP to bind in different modes to the active sites of the position 273 mutants. The productive binding mode for ASP is retained in the Thr^{273} and Ala^{273} mutant enzymes but other, nonproductive binding modes for ASP may become important. Hence, the K_m^{ASP} may increase for position 273 mutants enzymes because of competition between productive and nonproductive binding modes of ASP.

Interestingly, Val^{273} lies between and makes non-bonded contacts with the side chains of arginines 303 and 305 (Fig. 3). The mutation of Val^{273} to alanine should expand the range of allowable conformations of these argininyl side chains, through the elimination of non-bonded contacts. The mutation of Val^{273} to threonine may alter the conformations of arginines 303 and 305, as well. The side chain of Thr^{273} can hydrogen bond to Arg^{303}, but the rotation about its C^{α}-C^{β} bond necessary for this hydrogen bond would introduce bad steric contacts with Arg^{305}. Hence, the Thr^{273} active site, through its perturbation on arginines 303 and 305, could destabilize the productive recognition of ASP. Although position 273 undergoes little conformational change between ligated and unligated conformational states of the synthetase, all residues in contact with the side chain of position 273 exhibit large conformational excursions in response to ligand association. Hence, small energy changes in the interaction of the side chain at position 273 with the surrounding protein could well lead to significant conformational change.

The kinetic properties of the above mutations are clearly consistent with the absolute sequence conservation of valine at position 273. In fact, the selective pressures which retain position 273 as valine are now evident. *In vivo* concentrations of fumarate, succinate and ASP in *E. coli* are approximately 0.4, 0.4 and 2 mM (33,34). The mutations at position 273, presented here, are relatively conservative, yet each generates an enzyme which has a K_m^{ASP} at least 10-fold higher than intracellular concentrations of ASP and vastly improved binding affinities for succinate and fumarate. Under physiological conditions, the position 273 mutant enzymes are inactive.

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Table I
Kinetic paramaters for wild-type and mutant adenylosuccinate synthetases[a]

AMPSase	k_{cat} (s^{-1})	K_mGTP (μM)	K_mIMP (μM)	K_mASP (mM)	k_{cat}/K_mGTP ($s^{-1}\mu M^{-1}$)	k_{cat}/K_mIMP ($s^{-1}\mu M^{-1}$)	k_{cat}/K_mASP ($s^{-1}mM^{-1}$)
wild-type	1.00 ± 0.05	26.2 ± 2.3	27.8 ± 1.3	0.23 ± 0.04	(3.8 ± 0.4) x 10^{-2}	(3.58 ± 2.52) x 10^{-2}	4.4 ± 0.8
Thr^{128}→Ala[b]	0.46 ± 0.03	53.9 ± 6.1	307 ± 47	0.17 ± 0.02	0.85 ± 0.11	0.15 ± 0.03	2.71 ± 0.36
Thr^{129}→Ala[c]	0.42 ± 0.01	1700 ± 60	894 ± 73	0.24 ± 0.01	0.025 ± 0.0001	0.047 ± 0.0004	1.75 ± 0.08
Thr^{300}→Ala[d]	0.64 ± 0.04	165 ± 13	18.7 ± 1.2	6.17 ± 0.73	0.39 ± 0.04	3.42 ± 0.31	0.104 ± 0.014
Thr^{300}→Val	3.34 ± 0.17	55.8 ± 5.8	32.8 ± 2.8	0.82 ± 0.02	0.60 ± 0.07	10.1 ± 1.0	4.07 ± 0.23
Thr^{301}→Ala	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]	ND[e]
Val^{273}→Ala[f]	1.00 ± 0.1	31.1 ± 3.0	16.5 ± 1.8	7.36 ± 1.4	3.2 ± 0.5	6.1 ± 0.9	0.14 ± 0.03
Val^{273}→Asn[f]	0.05 ± 0.00	20.8 ± 1.9	23.6 ± 2.1	9.2 ± 2.1	0.24 ± 0.02	0.21 ± 0.02	0.0055 ± 0.001
Val^{273}→Thr[f]	1.99 ± 0.02	45.1 ± 6.5	43.1 ± 3.7	3.4 ± 0.2	4.4 ± 0.01	4.6 ± 0.6	0.61 ± 0.07

[a] Enzyme assay solution contained 20 mM Hepes, pH 7.7, 5 mM $MgCl_2$, and fixed concentrations for two of the three substrates, as appropriate, in the determination of K_m for the remaining substrate. The fixed concentrations of GTP, IMP and aspartate were 0.30, 0.50 and 5.0 mM, respectively, for wild-type AMPSase, and unless otherwise specified for the mutant AMPSases.
[b] 0.25 mM IMP and 0.45 mM GTP used
[c] 6 mM IMP and 0.60 mM GTP used
[d] 25 mM ASP used
[e] ND - Not Detectable/Not Determined
[f] 25 mM $MgCl_2$ used when varying ASP, 25 mM aspartate used

Table II

Kinetic parameters of wild-type and mutant AMPSases with NH_2OH[a]

AMPSase	k_{cat} (s^{-1})	K_m^{NH2OH} (mM)	K_m^{GTP} (μM)	K_m^{IMP} (μM)
wild-type	0.50 ± 0.09	230 ± 80	185 ± 22	66 ± 7
Thr^{301}→Ala	0.24 ± 0.04	91 ± 3	56.8 ± 6.8	316 ± 86
Val^{273}→Ala	0.21 ± 0.02	255 ± 60	142 ± 40	140 ± 20
Val^{273}→Asn	0.29 ± 0.08	109 ± 65	180 ± 30	113 ± 7
Val^{273}→Thr	0.080 ± 0.002	136 ± 34	74 ± 8	77 ± 6

[a]Performed as described in "Experimental Procedures".

Table III

Inhibition constants for wild-type and mutant AMPSases[a]

AMPSase	K_i^{SUC} (μM)	K_i^{FUM} (μM)	K_i^{MAE} (mM)
wild-type	890 ± 50	NI[b]	3.1 ± 0.8
Val^{273}→Ala	NI[b]	176 ± 40	1.0 ± 0.2
Val^{273}→Asn	NI[b]	42 ± 12	0.21 ± 0.08
Val^{273}→Thr	75 ± 11	640 ± 80	0.078 ± 0.005

[a]Performed as described in "Experimental Procedures"
[b]Non-Inhibitory (to 10 mM)

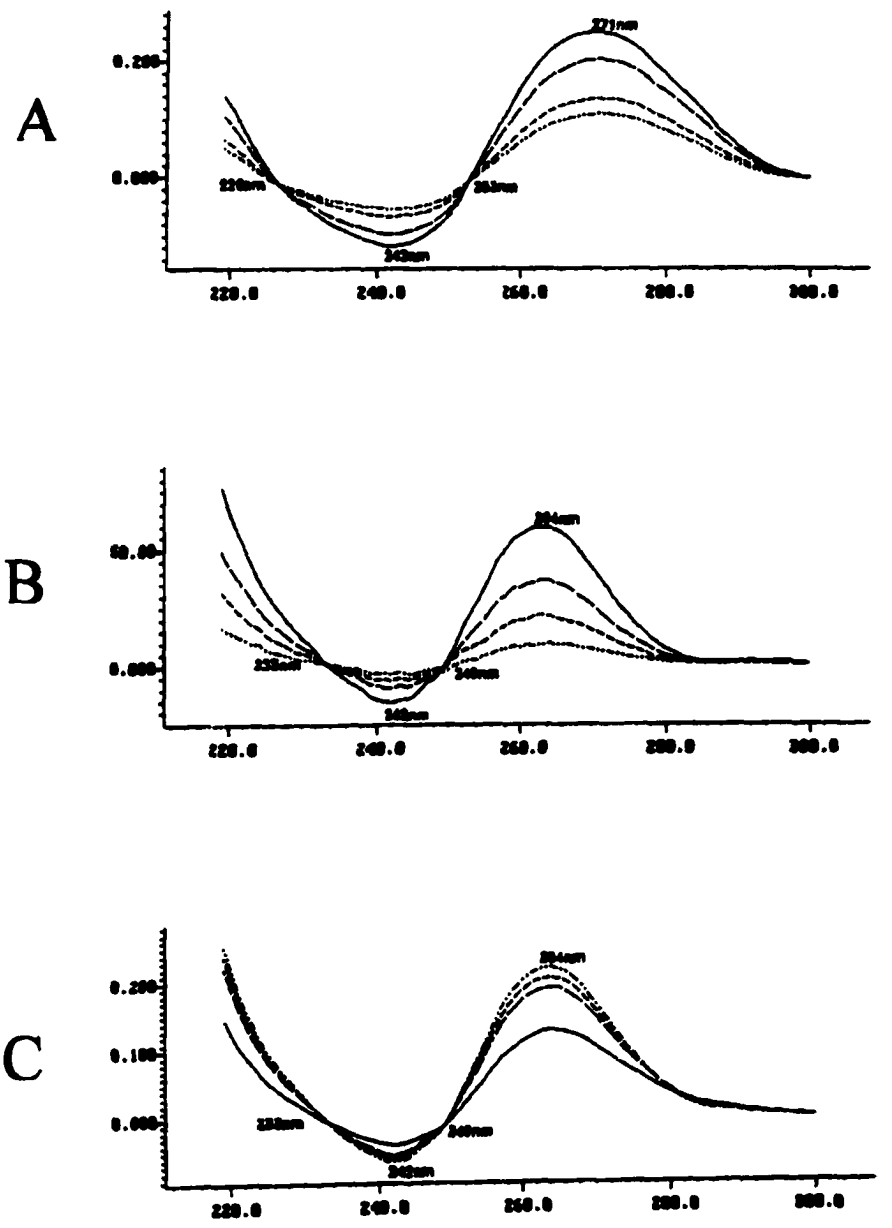

Figure 1 - UV absorbance difference spectra for reactions catalyzed by AMPSases using GTP, IMP Mg^{2+} and (A) aspartate as the substrate, (B) NH_2OH as the substrate, or (C) aspartate as the substrate, coupled with excess amount of adenylosuccinate lyase.

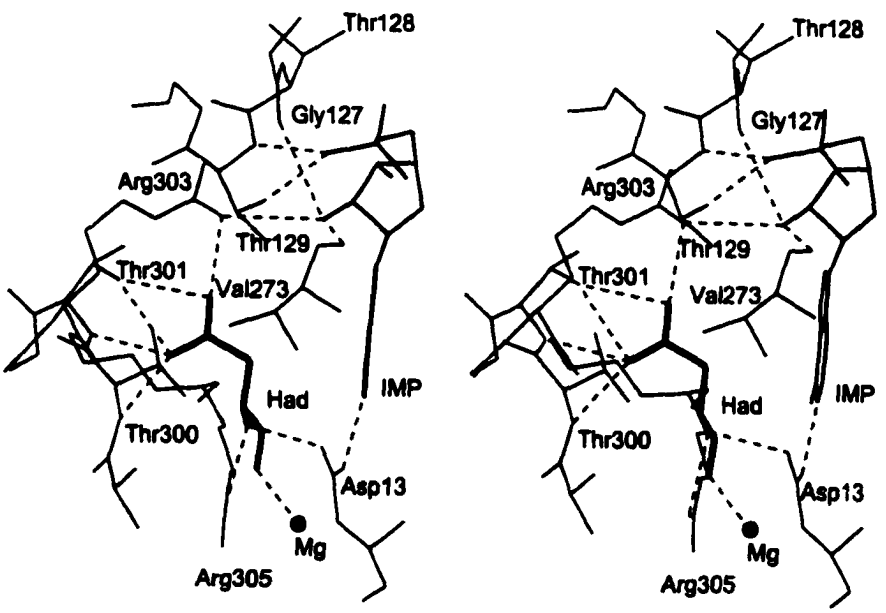

Figure 2 - Computer modeling of the IMP and hadacidin binding sites. The hydrogen bonds between enzyme and ligands are shown as dashed lines.

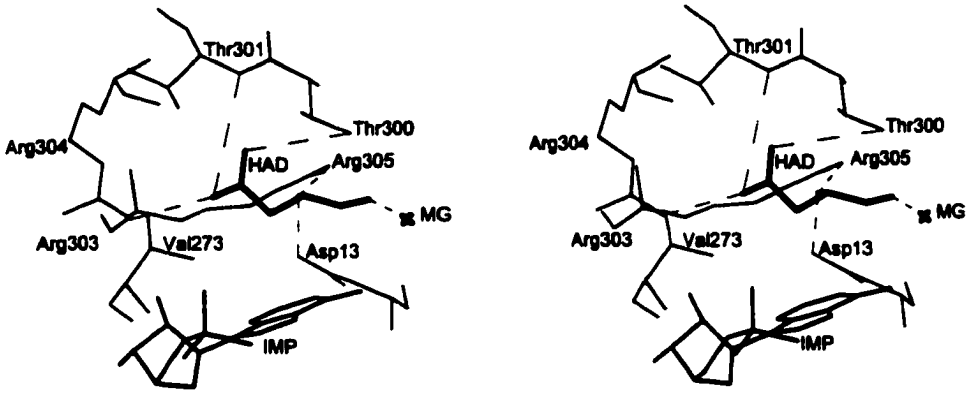

Figure 3 – Computer modeling of non-boning contacts between Val^{273}, Arg^{303}, and Arg^{305}

CHAPTER V. GENERAL CONCLUSION

Adenylosuccinate synthetase has been studied for over 40 years, starting with its initial discovery and elucidation of the catalyzed reaction, its placement in the purine nucleotide pathway, and tissue specific role in higher organisms. Since cloning of the gene, the details of the mechanics of how this enzyme works as a biological catalyst have been the focus of enzymatic studies. The importance of this enzyme lies in its place in the metabolism of purine nucleotides. It has been shown to be a target for natural antibiotics (1), herbicides(2), and for treatment of certain leukemias(3). In studies presented in this dissertation, the roles of certain residues in the function of the enzyme were examined, and the building blocks for the future exploration of this enzyme. Presented in this dissertation are the discussion of the state of association of the active form of the enzyme, and the difficulties which arise when using only directed mutations or crystal structures to assign roles to residues and their side chains.

First, the actual state of association of the enzyme was investigated. Monomeric and dimeric states of AMPSase had previously been reported in the literature (4,5), and all crystal structures of the enzyme are dimeric. Two residues which are located at the interface and implicated in dimer interactions from the crystal structures are Asp^{231} and Arg^{143}. Arg^{143} is a unique residue, as the side chain is involved in IMP binding at the active site of the symmetry-related monomer (Fig. 1b, Chapter II). Asp^{231} in the crystal structures forms hydrogen bonds with Arg^{147} and Lys^{140}, two residues which had previously been implicated by modification studies to be important in the activity of the enzyme (6,7). The mutations

Asp^{231}→Ala, Arg^{143}→Leu and Arg^{143}→Lys residues were made to disrupt the interface, so the importance of dimer interactions to enzyme activity could be determined.

When studied by MALDI, the wild-type enzyme showed the presence of both monomers and dimers. Sedimentation equilibrium analytical also showed the presence of both monomer and dimer states when performed in the absence of active site ligands. In the presence of ligands, the enzyme was exclusively a dimer, the wild-type AMPSase K_d drops from 11.2 μM to 1.03×10^{-38} μM (Table IV, Chapter II). In contrast, all of the mutant enzymes show only slightly lower K_d values when in the presence of ligands (Table IV, Chapter II), indicating the mutations do indeed affect the state of association. The k_{cat} values of the three mutants are comparable to that of the wild-type enzyme, however they show large increases in K_m^{IMP} (60- to 100-fold).

The enzyme exists as a monomer in the micromolar concentrations, which is the same range as the intracellular concentration of the enzyme. Binding of substrates shifts the monomer-dimer equilibrium to the dimer form of the enzyme. As this enzyme lies at the branch point from IMP in the *de novo* biosynthetic pathway, this ligand-induced dimerization may be an important regulatory mechanism in the control of the AMPSase reaction.

Chapter III investigates the roles side chains of amino acid residues 21, 38, 42 and 419 play in recognition and binding of the nucleotide IMP. Upon examination of the mutants, a number of unexpected results were obtained. In the crystal structure, Asp^{21} and Arg^{419} go from disordered conformations to form a salt-link in the ligated enzyme. In the ligated structure, Thr^{42} forms a salt-link with the guanine base, and the side chain of Asn^{38} appears to clearly form a hydrogen bond with the 5'-phosphoryl of IMP, and thus should be

important to the binding of IMP. Asp^{21} and Arg^{419} mutations are not located within any active site, yet mutation of either creates a drop in the k_{cat} of the mutants.

With the rapid equilibrium random mechanism, k_{cat} represents the breakdown of the quaternary complex of enzyme-substrate to enzyme-products, and is thus sensitive to changes in the transition state. Transition state stabilization yields an increase in k_{cat}, while destabilization yields a decrease in k_{cat}. The mutations discussed are not located in the active site, but in a substrate binding site, they must effect the active site though an indirect mechanism. This could be though either destabilization of the 40's loop, or through perturbations in the P-loop. Thr^{42} therefore also plays an indirect role, most likely though this 40's loop, as mutation causes approximately 10-fold increases in K_m^{GTP}.

Mutation of Asn^{38} to glutamine and aspartic acid shows that this residue plays no role in IMP binding (no influence on K_m^{IMP}). However, this residue is located in a short element which immediately precedes a loop (40's loop) which undergoes a 9 Å movement upon ligand binding. Binding of the substrate moves the 40's loop from an "open" position to a "closed" position (Fig. 2, Chapter III). As Asn^{38} plays no part in the binding of the substrate, the binding energy of this residue goes directly into moving the 'hinge' of a loop on the enzyme. These results help to remind us that a direct interaction seen in a crystal structure does not necessarily mean that it is required for the binding of the substrate, or that this is truly the function of that interaction.

Chapter IV further looks at the roles of direct and indirect interactions seen in the crystal structure on the binding of substrates. The study focused on a group of conserved β-branched side chains located near the 5'phosphate of IMP and carboxyl groups of L-aspartate. Residues Thr^{129} and Thr^{301} have direct interactions with IMP and L-aspartate,

respectively. Thr^{128} and Thr^{300} have indirect interactions with these same substrates (Fig. 2, Chapter IV). Thr^{128} interacts with substrates through contact with Asp^{114} in Loop 120 – 130, and the backbone carboxyl interacts with ASP in the case of Thr^{300}. Additionally, Val^{273} has no bonded contacts with ASP, though there are backbone interactions with Arg^{303} and Arg^{305} (Fig.3, Chapter IV), yet is conserved across all known AMPSases.

The effect on the steady-state kinetics by the mutant enzymes reflects the stabilization of the bound complexes by indirect hydrogen bonding as seen by the Thr^{129} and Thr^{300} mutants. Removal of these interactions by mutation to alanine causes an increase in the K_m^{IMP} and K_m^{ASP}, while causing only minor perturbations in the k_{cat} (Table I, Chapter IV). The effects of conformational restrains on the main chain of enzyme for L-aspartate recognition are seen from mutation of Thr^{300} to valine. The Thr^{300}→Val mutant enzyme retains conformational restraints on the backbone, but eliminates side-chain hydrogen bonds. This results in only a 3-fold increase in K_m^{ASP} and actually enhances k_{cat} by 300% (Table I, Chapter IV). As there is a naturally occurring valine in 25% of the known AMPSase sequences, this supports selection of either a theronine or valine in this position.

Direct interaction effects between substrates and side chains are seen for the residues 129 and 301. Thr^{129} forms a direct contact with the 5'-phosphoryl of IMP, while crystal structures show Thr^{301} hydrogen bonds with the carboxyl group of hadacidin through both its backbone amide and its side-chain, and additionally hydrogen bonds to Arg^{303} through its side-chain. Mutation of Thr^{129} to alanine only effects the K_m^{IMP} (Table I, Chapter IV). So unlike the mutations seen in Chapter III, only a small portion of the binding energy of the side-chain interactions, be they direct or indirect, for threonines 128 and 129 goes to the stabilization of the transition state (k_{cat} falls only twofold due to these mutations). The direct

interaction of Thr^{301} is essential for ASP binding, as when this interaction is removed, binding of ASP is removed. However the enzyme remains competent, as reaction occurs with hydroxylamine (Table II, Chapter IV).

The effect of a side-chain that from the crystal structure would not seem important is striking. What is the selective pressure to maintain a valine at this position, as is found in all known AMPSases? Inhibition of position 273 mutants by dicarboxylic acids provides a measure of insight as to how Val^{273} functions in substrate recognition. The mutants at this position show that there is an increase in the permissiveness of the biding site, seen in the drop of K_I for dicarboxylic acids. The intracellular concentrations of succinate and fumarate are sufficiently large enough to cause the Val^{273} mutant enzymes to be inactive under physiological conditions (8,9). This helps explain why there is a consistent valine at this position. The role of the non-bonding contacts is to prevent binding of nonproductive conformations of substrates, as seen by inhibition studies. The productive binding mode for ASP is retained in the Thr^{273} and Ala^{273} mutant enzymes but other, nonproductive binding modes for ASP may become important. Therefore, for position 273 mutant enzymes K_m^{ASP} may increase due to competition between productive and nonproductive binding modes of ASP.

The studies which were undertaken to investigate the roles of specific interactions in the substrate recognition of AMPSase yielded both expected and unexpected results, leading to insights for the mechanism of the enzyme. The results show the dangers of utilizing only a crystal structure or only a kinetic study to assign a role to a specific side chain or interaction, and these tools need to be utilized in conjunction to determine the function of a specific interaction or role of a side chain. Additionally, they highlight the fact that the interactions

deemed insignificant can play a larger role in the recognition of substrates, and clues as to their importance should not be overlooked.

Future direction in the study of *E. coli* AMPSase is to answer the question of the second magnesium which has been raised. Kang showed there are two Mg^{2+} found in the reaction(10), yet the presence of this second Mg^{2+} has yet to be seen in the crystal structure, while Honzatko proposed an alternative model where the second Mg^{2+} is between the a-carboxyl and ASP and Asp^{13} side chain. Additionally, a study on the differences between the isozymes which exist to see what gives rise to the differences needs to be done. To this end, a recombinant muscle isozyme has been produced. The enzyme has been characterized using both kinetic and biophysical techniques. Now that a recombinant form of the isozyme exists, the ability to examine what the structural basis for the difference between muscle/non-muscle and *E. coli* enzymes.

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